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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 34 retains the familiar format of previous volumes, this year with 33 chapters. Sections I - IV are disease-oriented and generally report on specific medicinal agents with updates from Volume 33 on antithrombotics, neurokinin receptor antagonists, anticoagulants, and new antibacterials. 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.

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 cytokines in T cell maturation, antibacterial screens, recombinant production of pharmaceutical proteins in plants, cell cycle regulation, and autoimmune diseases. 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 and parallel synthesis, molecular diversity and computational approaches to combinatorial chemistry, structure-based drug design, and the use of mass spectrometry in determining ADME properties in drug design.

Volume 34 concludes with To Market, To Market - a chapter on NCE and NBE introductions worldwide in 1998, a chapter on pharmagenomics and finally one on malaria as a third world disease in need of a first world drug development. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in volumes 1-34 of this series.

Volume 34 begins my first year as Editor in Chief of *Annual Reports in Medicinal Chemistry*. It is my pleasure to continue to work with highly professional and enthusiastic section editors. I would like to express my sincere thanks to Ms. Viviane Guillot and Ms. Lisa Bausch for their expert editorial assistance in assembling this volume.

Annette M. Doherty
Fresnes, France
May 1999

SECTION I. CNS AGENTS

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Chapter 1. Recent Approaches to Novel Antidepressant Therapy

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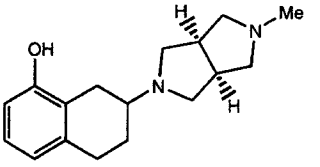
Introduction - The need for new antidepressants and approaches to antidepressant therapy is reflected in the volume and diversity of research that continues to be directed toward this important CNS therapeutic area (1). The call to develop "third generation antidepressants" has been sounded (2), but achievement of this goal to date has not been clearly demonstrated. Preliminary clinical results suggest some progress toward this goal, but the extent to which we have reached the next generation of antidepressant therapy is largely subject to interpretation. What is clear is that the three basic improvements in therapy that would constitute a third generation antidepressant, namely, faster onset of action, improved efficacy in refractory patients, and an improved side-effect profile, remain the primary targets for new antidepressant research (2). This chapter will highlight significant results in recent efforts to develop new pharmaceutical agents that could constitute a new generation of antidepressant therapy. Much research remains focused on the modulation of monoamine neurotransmitters (primarily serotonin and norepinephrine), guided by the monoamine theory of depression (3). Recently other approaches to therapy have also been pursued, with investigations of corticotropin releasing factor (CRF) antagonists and neurokinin antagonists being the most prominent (2, 4). Critical reviews of research surrounding these neuropeptides appear later in this volume, and these topics will not be covered in detail here.

MONOAMINE REUPTAKE INHIBITORS

Selective Serotonin Reuptake Inhibitors (SSRI's) - SSRI's have been available since the 1980's for the treatment of depression. The most widely known SSRI's are fluoxetine, sertraline, paroxetine, fluvoxamine, and citalopram (3). Although SSRI's have enjoyed wide clinical success, and possess a favorable side-effect profile relative to classical tricyclic antidepressants (TCA's), they are generally not more efficacious than the TCA's (4, 6). In addition, they exhibit a marked delay in onset of antidepressant action (generally 2-6 weeks), and are accompanied by their own particular set of side effects resulting from the non-selective stimulation of serotonergic receptor sites (7).

Reports of newer SSRI's have slowed in recent years, perhaps indicating a shift in interest to other approaches to developing new antidepressants. A recent survey of the antidepressant patent literature from 1995 to 1997 (4) reveals a number of interesting novel compounds with potent activity at the serotonin transporter (SERT), such as **1** (IC_{50} = 5.4 nM) and **2**



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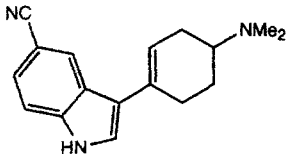
(IC_{50} < 10 nM). Heteroarylpiperidines **3** and halogenated naphthyloxypiperidines **4** (K_i = 0.014 nM, X = Br) have appeared in the patent literature more recently (8, 9).



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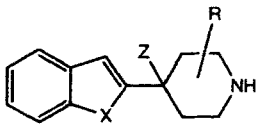

X = NH,S; Z = H, OH; R = H, Me

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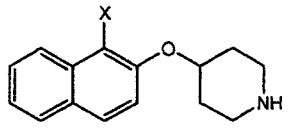
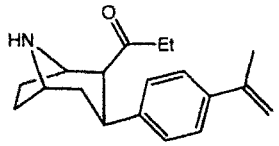


X = F, Cl, Br, I

4

Several reports of SAR studies on cocaine analogues have appeared (10-12). Cocaine itself is primarily a ligand for the dopamine transporter (DAT), but also has weak affinity for SERT and for the norepinephrine transporter (NET). Modification of the cocaine structure has led to a high affinity SERT ligand, **5** (IC_{50} = 0.11 nM), which is 145-fold and 854-fold selective over DAT and NET, respectively (10). The tropane **6** (IC_{50} = 0.69 nM) was also found to be selective for SERT (11). Interestingly, it has been found that the 8-amino function in the tropane is not required for binding to monoaminergic transporters, in contrast to the general assumption that an ionic bond is formed between the protonated amine and an aspartate residue on the transporter. The oxygenated derivative **7** displayed nanomolar potency at both SERT and DAT (12). Further SAR investigations on these non-basic transporter ligands could potentially lead to more selective SERT ligands.



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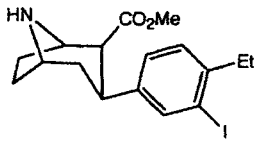

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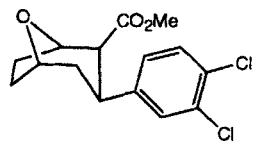

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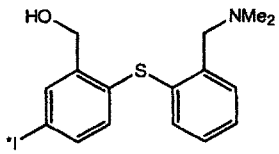
Single-photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging of the serotonin transporter in the living human brain have received attention as potential tools for understanding the relationship between alterations of the serotonin transporter and depressive illness (13). Radiolabeled ligands that have been prepared for this purpose include the iodine-123 analogue of **6**, which showed promise as a potential SPECT ligand in competition binding studies in mice (11). The diaryl thioether **8** displayed high binding affinity to SERT (K_i = 0.97 nM) and selectivity over DAT and NET; the iodine-123 analogue showed promise to be a superior SPECT imaging agent (13).



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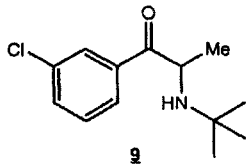
Norepinephrine Reuptake Inhibitors - Bupropion, **9**, is a clinically efficacious antidepressant whose mechanism of action is believed to be noradrenergic in nature (14). The 2-phenylmorpholinol **10** (BW 306U), a metabolite of bupropion, has been suggested to contribute to the drug's antidepressant profile. SAR investigation of **10** resulted in the identification of **11** (BW 1555U88), a selective inhibitor of norepinephrine uptake (IC_{50} = 70 nM). Compound **11** displays antidepressant activity in the anti-tetrabenazine rat behavioral model (14).



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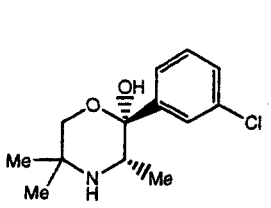

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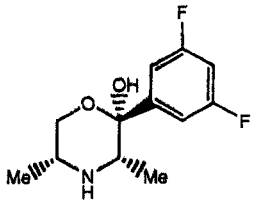

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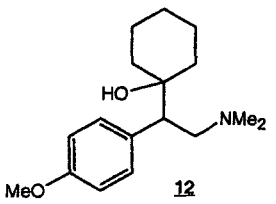
Serotonin / Norepinephrine Reuptake Inhibitors (SNRI's) - The SNRI venlafaxine, 12, has been on the market in the U.S. since 1993 for the treatment of depression (15). A recent clinical study has shown a significant beneficial effect on depression after one week of treatment, indicating a much faster response than has been reported before with antidepressant therapy (16). Duloxetine, 13, and milnacipran, 14, are also SNRI's that have reached clinical trial status (6).



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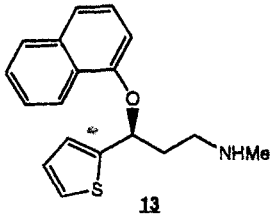

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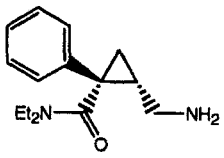

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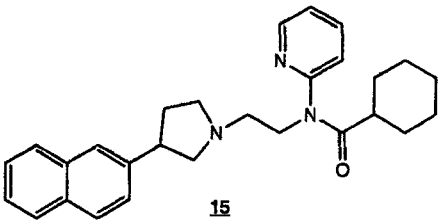
SSRI WITH 5-HT RECEPTOR MODULATION

SSRI + 5-HT1A - One approach to a faster-acting antidepressant that is receiving much attention is the combination of an SSRI with a 5-HT1A receptor ligand. This research is based on the hypothesis that the delay in onset of antidepressant action of an SSRI is the result of activation of the somatodendritic 5-HT1A autoreceptor. Activation of this presynaptic receptor by the increased levels of serotonin causes a decrease in neuronal firing, and hence no net increase in neurotransmission (7). Eventually, the autoreceptor is desensitized, and the length of time required for this process has been correlated with the time required to reach clinically therapeutic effects (17). Antagonism of the autoreceptor would block the reduced rate of neuronal firing, thus allowing the effects of the SSRI to be seen more rapidly. Evidence in support of this hypothesis has come from clinical trials using pindolol in combination with paroxetine (18). However, a study using pindolol and fluoxetine did not show significant reduction in the time of onset of clinical improvement (4).

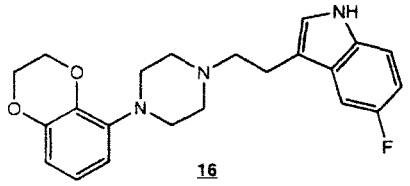
Efforts to identify molecules possessing both SSRI and 5-HT1A antagonist activities have resulted in a number of patents claiming mixed SSRI/5-HT1A antagonists, but little data on the *in vitro* or *in vivo* activity of such compounds is available. A notable exception is a series of arylcycloalkylamines that have been disclosed (19). The most promising example reported is the naphthyl-pyrrolidine, 15 (EMD 95750), which has 20 nM affinity for SERT and is also a potent (IC_{50} = 3 nM) 5-HT1A antagonist. Interestingly, the individual enantiomers display divergent affinities for the two



15

receptors; the (*S*)-enantiomer has higher transporter affinity than the (*R*)-isomer, while the opposite is true for the 5-HT1A affinity. Compound **15** and its enantiomers were reported to inhibit ultrasonic vocalization in rats, a model of anxiolytic activity, but no data is available on potential antidepressant activity (19).

A series of arylpiperazines having both SSRI and 5-HT1A antagonist activity, exemplified by **16** (SERT IC_{50} = 1.7 nM; 5-HT1A IC_{50} = 4.6 nM), have been reported (20). Pindolol derivatives, such as **17**, have also appeared (21).



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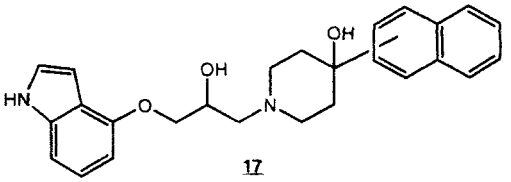

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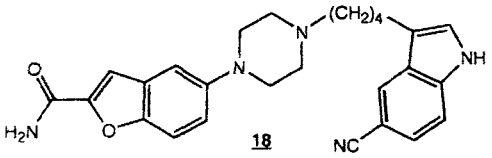
Compound **18** (EMD 68843) has been reported to be a potent SSRI (IC_{50} = 0.2 nM), and 5-HT1A agonist (IC_{50} = 0.5 nM), which exhibits antidepressant-like activity in rats, although it has a delayed onset of action after oral administration (22). Compound **18** was shown to be an agonist at the presynaptic 5-HT1A receptor site: it potently inhibited ultrasonic vocalization in rats, an effect that was counteracted by the 5-HT1A antagonist **19** (WAY-100,635). However, it had no *in vivo* effect at the post-synaptic site since it did not induce hypothermia, nor did it affect 8-OH-DPAT-induced hypothermia in rats (22).



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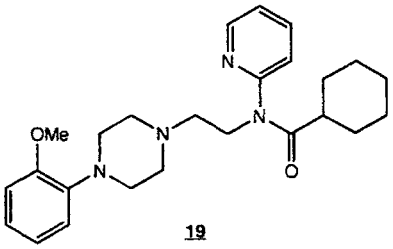
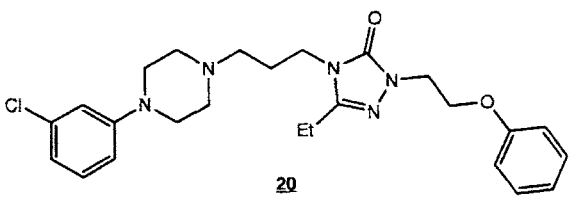


19

SSRI + 5-HT2 - It has been proposed that the interaction between 5-HT1A and 5-HT2 receptors may have implications in antidepressant therapy (23). 5-HT2 receptor antagonists reportedly enhance 5-HT1A receptor agonist-induced behavior, so that compounds having both SSRI and 5-HT2 receptor antagonistic activity might be expected to enhance serotonergic neurotransmission mediated by 5-HT1A receptors. 5-HT2 receptors are also believed to mediate many of the side effects of SSRI's, and therefore a combination SSRI/5-HT2 antagonist might be expected to have reduced side effects (4). Nefazodone, **20**, approved for the treatment of depression (U.S., 1995), is an example of a combination 5-HT2A antagonist and SSRI (24). A newer compound, **21** (YM-35992), which inhibits 5-HT uptake (IC_{50} = 21 nM) and is a 5-HT2A antagonist (IC_{50} = 86 nM), is active in the mouse tail suspension model of antidepressant activity (23). The indolyltetrahydropyridine **22** is a mixed SSRI/5-HT2A antagonist that is claimed to be superior, since it exhibits very low affinity for α1 and D2 receptors (25).



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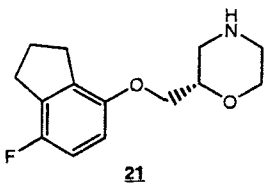

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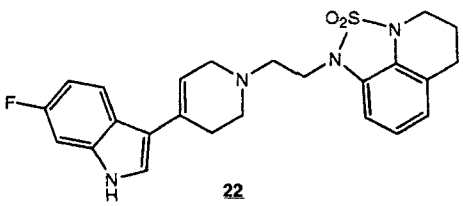
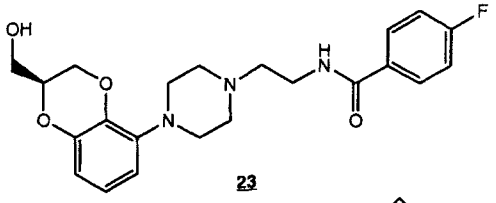


22

SEROTONIN RECEPTOR LIGANDS

5-HT1A - It is generally accepted that the 5-HT1A receptor plays a major role in psychiatric disorders such as depression and anxiety (7). There is evidence that 5-HT1A agonists or high efficacy partial agonists may be useful as antidepressants (4). The mechanism of action could be the result of a combination of agonist action at postsynaptic 5-HT1A sites combined with desensitization of presynaptic 5-HT1A autoreceptors. Flesinoxan, **23**, a selective 5-HT1A agonist, is in late-stage clinical trials for the treatment of depression (6).

Research efforts into selective 5-HT1A antagonists have been ongoing for a number of years; however, for antidepressant research, interest has focused on the use of 5-HT1A antagonists in combination with SSRI's, as discussed earlier. The most widely studied 5-HT1A antagonist is **19**, but its use in the clinic is limited by its rapid metabolism (26). Newer analogues of **19** have also been reported (17). In addition, combinations of SSRI's with the selective 5-HT1A antagonist **24** (NAD-299) and its derivatives have been claimed for the treatment of depression and other CNS disorders (27).



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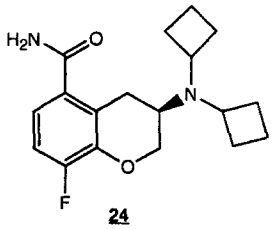
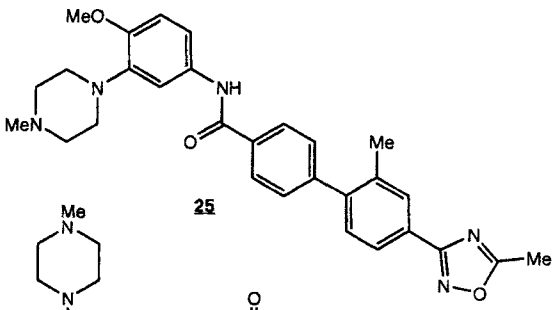


24

5-HT1B/1D - Blockade of terminal 5-HT1B autoreceptors (formerly known as 5-HT1Dβ) has been proposed as a new approach to developing potentially efficient and/or fast-acting antidepressants; acute autoreceptor blockade would, in theory, immediately elevate terminal 5-HT release (28). Compound **25** (GR-127935), reported to be the first example of a selective 5-HT1B/1D antagonist, has been found, more recently, to be a weak but full agonist at 5-HT1D and a partial agonist at 5-HT1B (28). Naphthylpiperazine derivatives such as **26** have been reported to be 5-HT1B antagonists. In particular, the *ortho*-tolyl analogue was shown to antagonize the inhibition of serotonin release induced by 5-carbamoyltryptamine in guinea pig hypothalamus slices (28).



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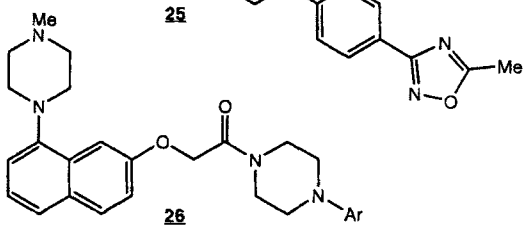

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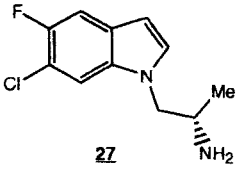
5-HT2C - Interest in developing 5-HT2C receptor ligands as potential psychotropic drugs has increased in the last few years. The exclusive CNS localization of the 5-HT2C receptor, with a high density in limbic and cortical regions of the brain, is consistent with a putative role in affective disorders such as anxiety and/or depression (29). A number of functional studies have provided evidence for the role of these receptors in the antidepressant action of marketed drugs, including TCA's, mianserin, trazodone, and fluoxetine, which were found to display moderate to high affinity for the 5-HT2C receptor. In addition, *meta*-chlorophenylpiperazine (*m*-CPP), an active metabolite of both trazodone and nefazodone, is considered to be a preferential 5-HT2C agonist (29).



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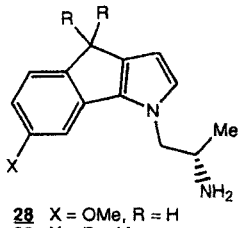
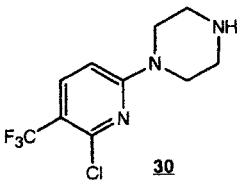


28 X = OMe, R = H
29 X = R = Me

Novel 5-HT2C receptor agonists have appeared in recent years. Compound **27** (Ro-60-0175, Org 38491), a 5-HT2C agonist (pK_i = 8.9) with 25-fold selectivity over the 5-HT2A receptor (30), displays anti-depressant effects in the anhedonia model of depression in rats (31). The tricyclic compounds **28** (Ro-60-0213, Org 35032) and **29** (Ro-60-0332) are both potent 5-HT2C agonists (pK_i = 9.0 and 8.5, respectively), with high selectivity over the 5-HT2A receptor (30). These have also been reported to be active in the rat anhedonia model (31).

Structurally unrelated compounds **30** (Org 12962) and **31** (Org 37684) display high affinity for the 5-HT2C receptor (32). Compound **31** (IC_{50} = 12 nM) is only slightly selective (5.4-fold) over the 5-HT2A receptor, while compound **30** (IC_{50} = 5 nM) is 64-fold selective. Both compounds were orally active in the penile erection model of 5-HT2C agonism, but no data was reported with respect to antidepressant-like activity (32).



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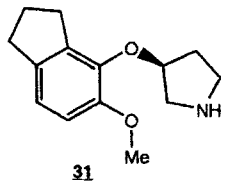

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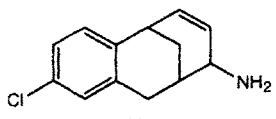
Other 5-HT2C agonists that have been disclosed recently include **32** (Org 8484), a compound identified through pharmacophore modelling and 3-D database searching (32). The constrained arylpiperazine **33** (pK_i = 7.6), although only a partial 5-HT2C agonist (60% intrinsic activity), is claimed to be active in the anhedonia model of depression in rats (33). The 5-HT2C agonist **34** (IC_{50} = 61 nM) is reported to have comparable affinity (IC_{50} = 72 nM) for SERT, and to exhibit high efficacy in mice in the forced swimming model of depression (34).



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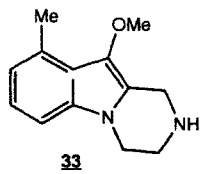

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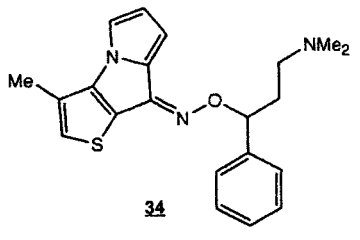

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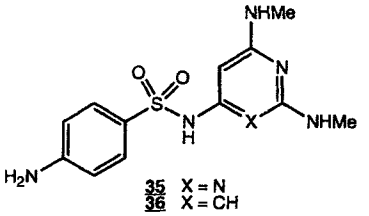
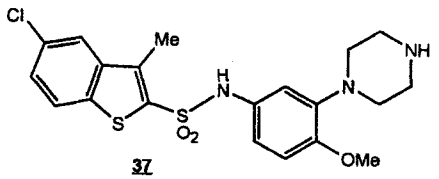
5-HT6 and 5-HT7 - The most recently cloned members of the serotonin family of receptors are the 5-HT6 and 5-HT7 receptors. Selective ligands for each of these receptors are required in order to fully evaluate the function and clinical potential of these receptors. It has been suggested that the 5-HT6 receptor may play a role in the treatment of depression, based on the receptor

distribution in the CNS and on the affinity of several antidepressant agents for this receptor (35). A similar argument has been made for the potential role of 5-HT7 in depression (4, 36). Compounds **35** (Ro-04-6790) and **36** (Ro-63-0563) are the first reported selective 5-HT6 antagonists (37). Compound **37** (SB-271046) has been identified more recently as a potent (pK_i = 8.9) and orally bioavailable 5-HT6 antagonist (35). The arylsulfonamide **39** and the ergot-like compound **38** are representative 5-HT7 antagonists appearing recently in the patent literature (38, 39).



35 X = N
36 X = CH



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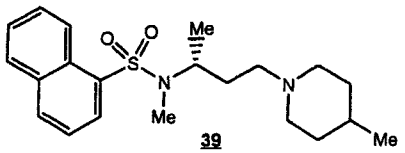

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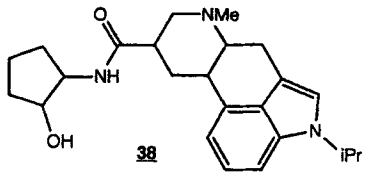

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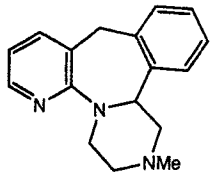
OTHER MONOAMINERGIC RECEPTOR LIGANDS

Mirtazapine **40**, the most recently approved antidepressant in the U.S., has been termed a "noradrenaline and specific serotonin antidepressant" or NaSSA (6). It has been reported to have a faster onset and better efficacy compared to SSRI's in comparator clinical trials; significantly greater improvement was seen at week two compared to citalopram, and at week one relative to paroxetine (16). Also, more patients responded to mirtazapine than to citalopram or paroxetine at all time points (16). The mechanism of antidepressant action is believed to be a result of mirtazapine's α2 antagonist activity, which results in increased release of both norepinephrine, via blockade of the α2 autoreceptor, and serotonin, via blockade of the α2 heteroreceptors located on serotonergic neurons (6). Mirtazapine has essentially no effect on dopamine, norepinephrine, or serotonin uptake. It is an antagonist at 5-HT2 (pK_i = 8.1), and 5-HT3 receptor sites, which may account for its lack of serotonergic side effects. However, it is not free from side effects, especially those attributable to activity at α1 and histamine H1 receptors (40).



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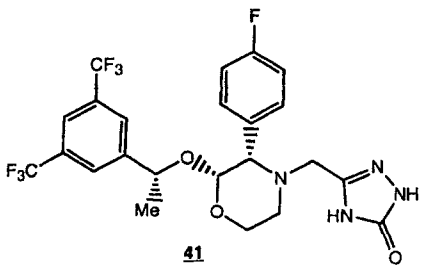
NON-MONOAMINERGIC RECEPTOR APPROACHES

Neuropeptides - Substance P has been known to be associated with inflammatory responses, the emetic reflex, and some CNS disorders such as Parkinson's disease and anxiety (41). Recent clinical trials of the NK-1 receptor antagonist **41** (MK-869) have raised the possibility that Substance P antagonists may also have antidepressant effects (42). Initial clinical results indicated that **41** showed promising antidepressant activity. However,



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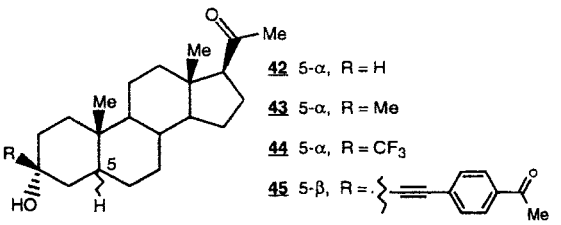
definitive efficacy was not demonstrated, possibly due to a high placebo response rate (43). NK-1 antagonists will not be discussed further here, as they are the subject of a separate chapter in this volume.

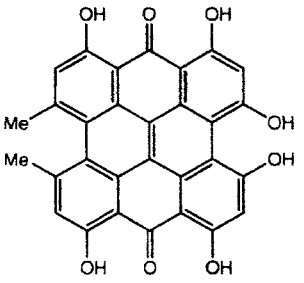
Corticotropin-releasing factor (CRF) is a neuropeptide which mediates the body's response to stress. Recent studies report increased levels of CRF in the cerebrospinal fluid of depressed patients, suggesting that abnormalities in CRF secretion may play a role in the pathophysiology of depression (44). CRF is also the subject of a separate chapter in this volume, and will not be further discussed here.

Neuroactive Steroids - The "epalons," named from the endogenous epiallopregnanolone, **42** (3α,5β-tetrahydroprogesterone; 3α,5α-THP), are a class of naturally occurring neuroactive steroids derived from the metabolism of progesterone. These compounds act as positive modulators of the $GABA_A$ inhibitory chloride channel complex, through binding to a novel allosteric site which is distinct from the barbiturate or benzodiazepine sites; they do not interact with the hormonal steroid receptors (45). Modulation of GABA receptors has been studied for several years in the context of depression (2). Recent reports have shown evidence from animal studies that fluoxetine increases levels of **42**, and clinical studies have shown that endogenous levels of epalons fall during depression, but are increased following successful antidepressant therapy (46). The semi-synthetic neurosteroids **43**, **44**, and **45** have displayed potent binding to, and positive modulation of, the $GABA_A$ complex (47-49). They have been claimed for use in a number of CNS disorders, including depression (4, 47). Clinical efficacy as antidepressants has not yet been demonstrated, but remains an intriguing possibility.



NATURAL PRODUCTS

Hypericum - The top-selling antidepressant in Germany is an herbal remedy, derived from *Hypericum perforatum*, commonly known as St. John's wort (4). The mechanism of antidepressant action of *Hypericum* extracts is not understood, although a recent study on standardized extracts, such as LI-160, demonstrated a dose-dependent inhibition of both serotonin and norepinephrine uptake in astrocytes (50). The active component of the extracts may be hypericin **46**, but up to ten active constituents could be involved. It has been suggested that hyperforin, **47**, could be more active that hypericin (51). Clinical trials for depression using LI-160 extracts are being planned in the U.S. (51).



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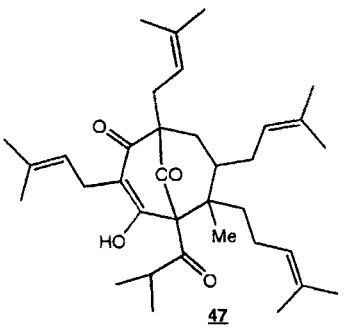

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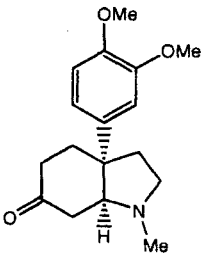
Mesembrine - The alkaloid (-)-mesembrine, **48**, is found in plants of the *Mesembryanthemaceae* family. A recent patent claims the use of **48** and related compounds for the treatment of depression (52). These compounds are reported to be serotonin reuptake inhibitors; **48** has a binding affinity of 36 nM for SERT (52). The use of various plants from the *Mesembryanthemaceae* family in psychoactive preparations has been reviewed (53).



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Conclusion - Significant progress has been made in the search for "third generation antidepressants," which would be characterized by a faster onset of action, reduced side-effect profile, and efficacy in refractory patients. However, no agent has yet been identified which definitively meets all three criteria. Much research remains directed at new approaches falling within the general scope of the monoamine theory of depression, including recent interest in the modulation of post-receptor signal transduction pathways (54). Research into novel pathways that have been implicated in the pathophysiology of depression, including the role of neuropeptides and neuroactive steroids, may lead to promising new antidepressants. At the present, however, the therapeutic utility of modulating these pathways has yet to be proven.

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Chapter 2. Recent Progress in Corticotropin-Releasing Factor Receptor Agents

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Introduction - Corticotropin-releasing factor (CRF) is a neurohormone which appears necessary and sufficient for the organism to mount functional, physiological and endocrine responses to stressors (1). Factors which mobilize brain CRF systems appear to have one feature in common, -the ability to disturb homeostasis. For example, demands on the organism may be induced either internally or externally by exposure to physical trauma, infection or social conflict. Coping responses to such deflections in steady state which include sympathetic nervous system activation, promotion of negative energy balance and augmentation of vigilance and emotionality appear to be CRF-dependent. Not surprisingly, many human psychopathologies which include hyperexcitability or anxiety-like components are hypothesized to depend either causally or symptomatically on overactivation of CRF in brain. This chapter will document recent progress towards the goal of assigning functional significance to endogenous CRF circuits in brain, and examine the state of the art regarding the growing array of pharmacological tools, including the most recently published small-molecule ligands, which are available for further probing the physiological significance of CRF system activation.

FUNCTIONAL SIGNIFICANCE OF CRF RECEPTORS AND CRF-BINDING PROTEIN

CRF Receptors – CRF receptors belong to the recently described family of "gut-brain" neuropeptide receptors. Other typical members of this family include receptors for calcitonin, vasoactive intestinal peptide, parathyroid hormone, secretin, pituitary adenylate cyclase-activating peptide, glucagon and growth hormone-releasing factor. All of these receptors possess seven putative transmembrane domains and are positively coupled to adenylate cyclase.

The CRF_1 receptor was first cloned from several species including human (2, 3), mouse (3) and rat (4, 5). Species homologs are 98% identical over their full length of 415 amino acids. In general, the CRF_1 receptor is approximately 30% identical to all other members of the neuropeptide receptor family. Characteristic of most G-protein coupled receptors, the CRF_1 receptor has putative N-linked glycosylation sites on the N-terminal extracellular domain. There are five predicted sites on CRF_1, substantiating the glysosylation profiles determined by chemical affinity cross-linking studies (6). In addition, there are potential protein kinase C phosphorylation sites in the first and second intracellular loops and in the C-terminal tail, as well as casein kinase II and protein kinase A phosphorylation sites in the third intracellular loop (2).

There are currently three known forms of the CRF_2 receptor: $CRF_{2\alpha}$, $CRF_{2\beta}$ and $CRF_{2\gamma}$. The $CRF_{2\alpha}$ receptor, which was originally described by Lovenberg et al. (7), is a 411 amino acid protein with approximately 71% identity to the CRF_1 receptor. The

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$CRF_{2\beta}$ receptor, which has been cloned from both rat (7) and mouse (8, 9), is 431 amino acids in length and differs from $CRF_{2\alpha}$ in that the first 34 amino acids in the N-terminal extracellular domain are replaced by 54 different amino acids. A third splice variant, the $CRF_{2\gamma}$ receptor, has recently been identified in human brain (10). This splice variant uses yet a different 5' alternative exon for its amino terminus and replaces the first 34 amino acid sequence of the $CRF_{2\alpha}$ receptor with a unique 20 amino acid sequence. RT-PCR analysis of human brain mRNA demonstrated expression in amygdala and hippocampus while southern analysis of rat genomic DNA yielded negative results, suggesting that this subtype does not exist in rat. All splice variants of the CRF_2 receptor have potential N-glycosylation and phosphorylation sites, which are analogous to those found in CRF_1 receptors. It is interesting to note that there are very large regions of amino acid identity between CRF_1 and CRF_2 receptors, particularly between transmembrane domain five and transmembrane domain six. This similarity argues strongly for conservation of biochemical function since it is this region which is thought to be the primary site of G-protein coupling and signal transduction.

CRF Binding-Protein (CRF-BP) - Plasma CRF is substantially elevated during the third trimester of human pregnancy and this process is likely to participate in a cascade of events which eventually leads to parturition (11). It was subsequently demonstrated that the majority of this late gestational maternal plasma CRF is bound to a high affinity CRF-binding protein (CRF-BP) which neutralizes the ability of CRF to release adrenocorticotropic hormone (ACTH). Thus, the levels of CRF-BP in the maternal plasma determine the amount of 'free' CRF that is available to interact with pituitary CRF receptors and thereby modulate the activity of the pituitary-adrenocortical axis during late human pregnancy. The predominant tissues expressing CRF-BP in all species are the brain and the pituitary gland where the protein is hypothesized to regulate CRF actions (12). With respect to the CNS and the role of CRF-BP, it has recently been demonstrated that CRF-BP is expressed in various areas of brain including the cerebral cortex, amygdala, hippocampus, hypothalamus as well as sensory relays associated with the auditory, olfactory, vestibular and trigeminal systems. Of note, there are brain areas that are enriched with CRF and CRF-BP but have only low densities of receptors and conversely, other brain areas which are enriched with receptors and devoid of CRF-BP (13). Thus, the differential distribution of brain CRF-BP and CRF receptors presents multiple distinct sites of interaction with CRF for potential exploitation in the treatment of central deficits in CRF neurotransmitter function.

CRF Mutant Mouse Models – The profusion of CRF peptide, CRF post-synaptic receptor and CRF binding-protein transgenic and knockout mouse models reported over the past year allows for critical analysis of hypotheses relating pituitary-adrenocortical axis tone and brain CRF system activation in animal models to a variety of clinical psychopathologies. One phenotype common to all of the CRF mutants is altered tone/reactivity of the pituitary-adrenocortical axis. CRF overexpressor (transgenic) mice exhibit an overabundance of CRF in brain as well as 5-10 fold elevations in plasma levels of ACTH and corticosterone (14). CRF-knockout mice which are CRF deficient lose normal circadian variations in plasma ACTH and corticosterone which are restored by constant infusion of exogenous CRF (15). CRF_1

receptor knockout mice display low basal levels of plasma corticosterone and attenuated ACTH and corticosterone stimulation in response to a restraint stressor (16). CRF binding protein transgenic mice which exhibit a normal pattern of basal and stimulated plasma ACTH and corticosterone levels appear to counterbalance the consequences of CRF binding protein enrichment via a compensatory 82% increase in expression of CRF (17). It is important to note that while the CRF mutants accommodate their genetic re-programming by adaptation of the endocrine stress axis at different set points, each mutant retains the ability to activate the pituitary-adrenocortical axis, albeit in diminished or altered fashion, in response to stress.

The putative role of brain CRF in affective disorders has been examined using CRF_1 receptor knockout mice and CRF binding-protein overexpressing and knockout mice. The phenotype of CRF mutants with under- or over-activation of endogenous CRF systems can now be compared to the expected consequences of stress-axis under- or overactivation predicted by clinical findings (18). For instance, CRF overexpressor mice exhibit an anxiogenic-like phenotype characteristic of a fight or flight response, whereas deactivation of brain CRF systems via CRF neuropeptide knockdown, CRF_1 receptor knockdown or knockout evokes complementary, anxiolytic-like attributes (19). Bioneutralization of CRF by an indirect means, overexpression of CRF-BP which complexes and neutralizes endogenous CRF, also produces anxiolytic-like properties *in vivo*. Elucidation of affective phenotypes for CRF and CRF_2 receptor knockout mice will soon be possible. The positive correlation between CRF levels and emotionality in these animal models is in keeping with the hypothesized role of CRF in mediating human affective diseases.

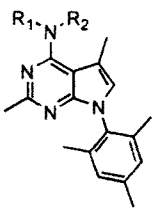
CRF Gene Knockdown - Gene knockdown, also referred to as antisense oligonucleotide targeting, suppresses expression of a particular gene product via translational arrest. Thus, the *in vivo* consequences of gene knockdown would be expected to be consistent with the pattern of functional effects of gene deletion or administration of competitive receptor antagonists. Treatment of rats with an antisense directed against CRF was shown to produce anxiolytic-like effects. Compared to vehicle-treated animals, lower ACTH and corticosterone levels were also observed in the antisense-treated animals following exposure to a shuttle-box avoidance procedure (20). Recent antisense studies focused on CRF_1 and anxiety-related behaviors in particular have demonstrated that treatment with CRF_1 antisense attenuates the anxiogenic-like effects induced by central administration of CRF (21). Consistent with this finding, chronic infusion of a CRF_1 antisense into the central nucleus of the amygdala was also reported to reduce anxiety-related behaviors in socially defeated rats (22). In contrast, treatment with antisense directed against CRF_2 receptors did not affect performance in the defensive withdrawal paradigm, thus suggesting that CRF_2 may not participate in the neural pathways mediating anxiety-related behaviors (23).

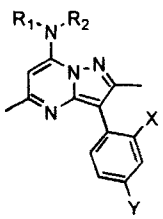
Human Stress-Axis Pathophysiology - In rodent animal models, central administration of CRF has been demonstrated to produce anxiety-like effects, decreased food consumption, diminished sexual behavior and altered sleep patterns (24). These behavioral changes closely parallel signs and symptoms observed in human psychiatric disorders including major depression, anxiety disorders and anorexia

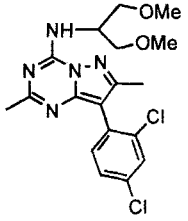
nervosa, thus suggesting a role for CRF in the pathophysiology of mental illness. Accordingly, CRF hypersecretion has been detected in a large portion of individuals diagnosed with major depression (18). In depressed patients, elevated cortisol levels and a blunted ACTH response to CRF administration were observed, thus supporting the notion that the hypercortisolism observed in major depression is due to abnormal CRF secretion in the central nervous system. Noteworthy, treatment of depressed patients with antidepressant drugs or electroconvulsive therapy resulted in a decrease of the altered levels of CRF observed before the antidepressant treatment as well as in improvements in the clinical conditions of these patients. This observation strengthens the hypothesis that overactivation in brain CRF pathways may underlie certain features of the symptomatology seen in affective disorders, in which case CRF receptor antagonists would be expected to have a therapeutic benefit. In complementary fashion, CRF receptor agonist administration could conceivably counteract the consequences of diminished activation of brain CRF circuits (25).

CRF RECEPTOR ANTAGONISTS, AGONISTS AND CRF-BP LIGAND INHIBITORS

Non-peptide CRF_1 receptor antagonists that can selectively block the CRF_1 receptor subtype inhibit CRF-stimulation of cAMP or CRF-stimulated ACTH release from cultured rat anterior pituitary cells (26). Furthermore, when administered peripherally, these compounds compete for *ex vivo* [^{125}I]sauvagine binding to CRF_1 receptors in brain sections demonstrating their ability to cross the blood-brain-barrier. Peripheral administration of these compounds attenuates stress-induced elevations in plasma ACTH levels demonstrating that CRF_1 receptors can be blocked in the periphery. Furthermore, peripherally administered CRF_1 receptor antagonists have also been demonstrated to inhibit CRF-induced seizure activity (27) suggesting that non-peptide CRF_1 receptor antagonists, when administered systemically, also specifically block central CRF_1 receptors. Extensive evidence validates the ability of CP-154,526 (**1**) to competitively antagonize CRF_1 receptors. This compound attenuates fear-potentiated startle after oral administration in rats (28) and in the same study the oral bioavailability of **1** was estimated to be 37%. Comparison of the inhibition of binding of 70 pM [^{125}I]Tyr^0-o-CRF to rat brain by the monobutyl analog (**2**) (IC_{50} = 110 nM) and monoethyl analog (**3**) (IC_{50} = 620 nM) of **1** (IC_{50} = 5.5 nM) was made. A synthetic route to a ditritiated analog of **1** is now available (28).



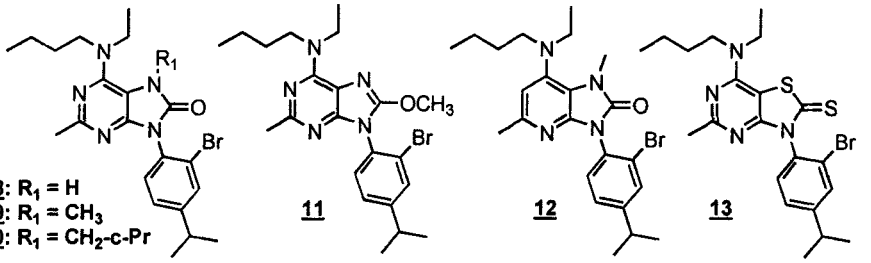




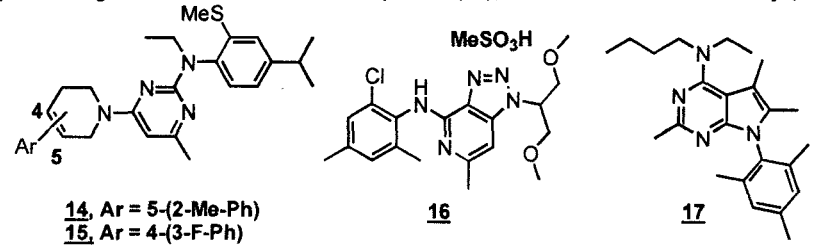
1: R_1 = n-Bu, R_2 = Et
2: R_1 = n-Bu, R2 = H
3: R_1 = H, R_2 = Et
4: R_1 = H, R_2 = $CH(Et)_2$, X = CH_3, Y = OCH_3
5: R_1 = CH_2-c-Pr, R_2 = n-Pr, X = Y = Cl
6: R_1 = n-Pr, R_2 = $CH_2CH_2OCH_3$, X = Y = OCH_3
7

The efforts of several groups have focused on the synthesis of pyrazolo[1,5-a]pyrimidines as CRF receptor antagonists. After the publication of the first patent application (29), two additional world patent applications appeared (30, 31). The pharmacokinetics in dog of SP904 (**4**) (Ki = 1 nM) showed $t_{1/2}$ (po) = 45 hours and oral

bioavailability of 33.1 % (32). A SAR study of similar compounds found the most potent analog, **5**, to contain a 2,4-dichlorphenyl ring and a N-propyl N-cyclopropylmethyl amino side chain at the 7-position (Ki = 3.2 nM) (33, 34). From a SAR study, NBI 30545 (**6**) was selected for further study (Ki = 2.8 nM) and demonstrated efficacy in the CRF-induced locomotor activity model in mice (35). DMP-696, (**7**), (Ki = 1.7 nM) an orally active CRF receptor antagonist from the related pyrazolo[1,5-a]-s-triazine series (30), demonstrated 50% oral biavailability in dogs with $t_{1/2}$ = 33.4 hours and $t_{1/2}$ = 15 hours in rhesus monkeys (p.o.). No endocrine, cardiovascular, GI, or pulmonary effects were noted at doses 30-fold higher than those effective in the rat situational anxiety assay. Furthermore, the potency of the molecule improves on chronic administration (36).

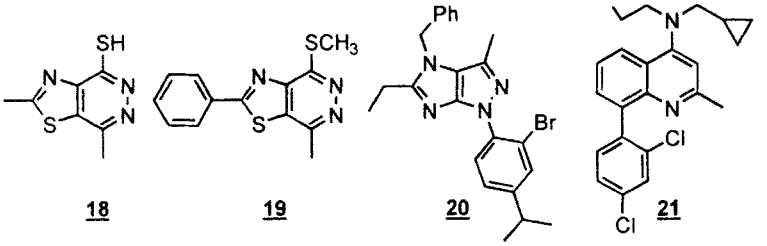


Certain N-7-alkyl-N-9-aryl-8-oxopurines, as well as structurally related imidazopyridine-2-ones, are CRF_1 receptor antagonists (37). The importance of a small N-7 alkyl group for tight binding of 8-oxopurines to the CRF_1 receptor is seen by comparing **8** (Ki = 890 nM), **9** (Ki = 4.9 nM) and **10** (Ki = 117 nM). O-Methylation of the 8-oxo group on **8**, to yield **11**, provides a more potent antagonist, (Ki = 1.5 nM) while the 1-deaza analog **12** (Ki = 4.0 nM) was equipotent to **9**. Thiazolo[4,5-d]pyrimidine thione **13** (Ki = 4.1 nM) contains the favored 2-bromo-4-isopropyl substituted phenyl ring common to a number of tight binding CRF receptor antagonists. CRA0165 (**14**) (IC_{50} = 12.7 nM) and CRA1000 (**15**) (IC_{50} = 10.5 nM) were found to have good affinity for the CRF_1 receptor. Oral administration of these compounds to mice and rats with stress-induced and CRF-induced anxiogenic-like activity ameliorated these effects in the 0.1 to 10 mg/kg range. Several anilino-aminopyrimidine-based CRF receptor antagonists have previously been reported (38-41). DMP 695 (**16**), obtained from the N-aryl aminotriazolopyrimidine series of CRF receptor antagonists and described in a patent (42), demonstrated oral activity (43).



While a wide variety of anti-stress, anxiolytic and anti-depressant actions of small molecule CRF_1 receptor antagonists have been recently reported (44), one example which has received much attention concerns the fact that regulation of CRF levels may correlate with the length of pregnancy (45). In particular, plasma CRF levels increase exponentially as gestation advances beyond about 16 weeks and could possibly serve as a good predictor of delivery. Noting that women with the highest CRF levels were most likely to deliver prematurely, these investigators have posulated that CRF receptor blockade would act to delay parturition. In support of this hypothesis, infusion of antalarmin (**17**) to pregnant sheep delays delivery of lambs

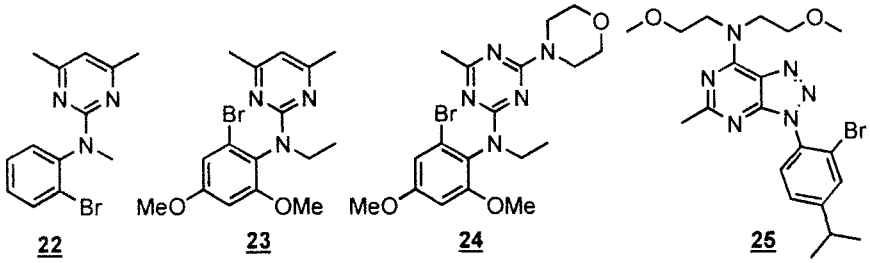
until infusion is discontinued (46). Thiazolopyridizines **18** and **19** were reported by the same group to be promising leads in the development of CRF receptor agonists.



18 **19** **20** **21**

The imidazolo[4,5-c]pyrazole **20** (Ki = 4 nM) is the most potent CRF receptor antagonist in a SAR study around this 5-5 bicyclic series (47). A SAR study on the contrasting 6-6 bicyclic 8-arylquinolines, represented by **21**, (Ki = 0.5 nM) reported a number of analogs with Ki values under 1 nM (48).

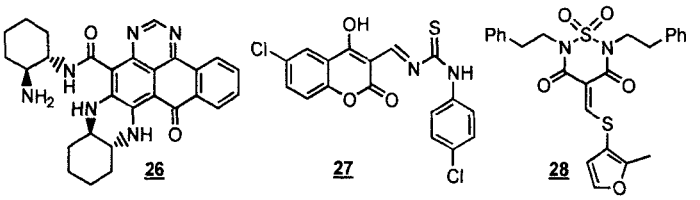
The screening lead **22** (Ki = 5700 nM) was optimized by systematic SAR studies that assisted in the definition of a pharmacophore (49) that is similar to a model previously proposed (38). Conformational preferences and barriers to rotation of anilino-aminopyrmidine **23** were determined by semiempirical methods, X-ray and variable temperature NMR spectroscopy. The study determined the active conformation of the anilinopyrimidines and led to the synthesis of additional anilino-amino-based pyrimidine CRF receptor antagonists as well as SA627 (**24**) (Ki = 32 nM) (50). Compound **24** was evaluated in dog at 5 mg/kg (iv, po). The oral bioavailability was 20 % and the mean peak oral plasma level was 730 nM at 0.5 hours. However, this compound was not advanced further since members of the bicyclic triazolo[4,5-d]pyrimidine series were more promising. From an extensive SAR study, SC241 (**25**) (Ki = 3.7 nM) was believed to have properties necessary for a potential therapeutic agent. Further pharmacological studies are planned for this compound (51).



22 **23** **24** **25**

In the past two years, since the last review on CRF in this series (26), the number of patents and applications combined has nearly doubled. However, there is a scarcity of receptor binding data published in these patents. Two reviews have appeared that include these patents (44, 52). The more recent review summarizes generic structures and gives examples of structures claimed for all the small molecule CRF receptor antagonist patents (44).

Oxo-7H-benzo[e]sperimidine-4-carboxamide **26** displaced radiolabeled o-CRF from both CRF_1 (Ki = 110 nM) and $CRF_{2\beta}$ (Ki = 20 nM) receptors (53). Compound **26** is the most potent analog in a series of the first reported mixed CFR_1/CRF_2 receptor antagonists. The compound antagonized CRF-stimulated cAMP formation and CRF-stimulated corticotropin release from rat pituitary *in vivo*, suggesting an antagonist. The binding selectivity profile was determined for **26** at a number of different receptors. The compound showed weak binding to NPY Y1 (Ki = 4200 nM), D_2 (Ki = 3200 nM) and 5-HT7 (Ki = 1600 nM), but binding > 10,000 nM against the other receptors screened.



26 **27** **28**

The first patent application for CRF binding protein ligand inhibitors claiming structure **27**, as well as related compounds, has recently appeared (54). Rats were treated with 1 mg/kg (po) of **27** and were subjected to the Morris Water Maze test. A slight improved performance was reported over control rats after three days of acquisition training. In a second patent application (55), compound **28** and related structures, were claimed as CRF binding protein ligand inhibitors. No biological data appeared in the patent application.

CRF RECEPTOR AND CRF-BP SELECTIVE PHARMACOLOGICAL TOOLS

Construction of CRF Peptide Analogs – The native rat/human CRF(1-41) peptide affords a variety of opportunities to study CRF neurobiology by virtue of the fact that discrete and separate sequences of amino acids within the peptide chain subserve CRF receptor binding, receptor signaling and CRF-BP binding functions (56). For instance, the cyclization of shortened agonists of CRF at positions 30–33 to form a lactam ring (e.g. cyclo(30-33)[Ac-Leu^{8},DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF(8-41) (or the equivalent positions 29-32 in sauvagine and urocortin) induces an α-helical constraint that stabilizes a bioactive conformation of the peptides. In addition, evidence was presented that the leucine/isoleucine residues at position 8 of CRF peptide agonists bears sole responsibility for activation of the receptor (57). Similarly, the tertiary structure of CRF necessary to bind its postsynaptic receptors is provided by the aminoterminal region of the peptide whereas amino acids near the carboxyterminus are necessary for binding CRF-BP (58). These inherent physiochemical properties allow endogenous CRF family agonists such as r/h CRF(1-41) and rat or human urocortin (1-40) to be modified forming peptides such as α-helical CRF(9-41) or d-Phe CRF(12-41) which are competitive receptor antagonists and peptides such as r/h CRF(6-33) which is a selective CRF-BP ligand inhibitor (Table 1). This same approach may also provide receptor selective agonist peptides (59) which can guide the discovery of small molecule agonists such as **18** and **19**.

In Vivo Efficacy Profile of CRF Receptor and CRFBP Ligands – The differential binding profile for CRF family ligands exhibited in Table 1 is mirrored by distinct functional actions following administration in animal models. For example, the competitive CRF receptor antagonists, α-helical CRF (9-41) and d-Phe CRF (12-41) both block CRF_1 and CRF_2 receptors and yet are easily distinguished *in vivo* by the fact that α-helical CRF (9-41), but not d-Phe CRF (12-41), produces partial agonist actions (60) which may be due to affinity of α-helical CRF (9-41) for the CRF binding-protein. This supposition is supported by functionally selective *in vivo* effects of an indirect CRF agonist, the competitive CRF binding-protein ligand inhibitor peptide rat/human CRF (6-33), relative to the profile of a full post-synaptic CRF_1/CRF_2 receptor agonist such as CRF itself (61). Similarly, the rank order in affinity of urocortin (CRF binding-protein > CRF_2 receptor > CRF_1 receptor) favors a functional dissociation from CRF itself which has a slightly different rank order profile (CRF binding protein > CRF_1 receptor > CRF_2 receptor). Consistent with this interpretation, the dissociation kinetics of CRF and urocortin from CRF binding-protein reveal an irreversible binding profile for urocortin (62) which could account, in part, for differential *in vivo* efficacy of urocortin versus CRF reported in some studies (63). Urocortin has also been examined in a variety of rodent behavioral and physiological assays of motor activity, exploratory inhibition, cardiovascular tone, malaise and food intake.

These paradigms were selected for their sensitivity to central nervous system and physiological activation states induced by exposure to environmental stressors and exogenous administration of CRF-like peptides such as CRF itself, sauvagine and urotensin. Anorexic actions of urocortin *in vivo* appear to be analogous to those of CRF, although urocortin has been reported to be the more potent of the two agonists in reducing food intake in some (63) but not all (64) studies. Thus, the precise neurobiological substrates for *in vivo* actions of CRF-family neuropeptides will likely follow extensive pharmacological studies employing CRF receptor subtype and CRF binding-protein selective ligands.

Table 1. Binding Profile of CRF Family Ligands

Ligand	CRF_1	CRF_2	CRF-BP
r/h CRF(1-41)	**Yes**	**Yes**	**Yes**
r/h urocortin (1-40)	**Yes**	**Yes**	**Yes**
r/h CRF(6-33)	No	No	**Yes**
Non-peptide CRF-BP Ligand Inhibitor (**27**,**28**)	No	No	**Yes**
o α-helical CRF(9-41)	**Yes**	**Yes**	**Yes**
r/h d-Phe CRF(12-41)	**Yes**	**Yes**	No
CP-154,526 (**1**)	**Yes**	No	No
Non-peptide CRF_1/CRF_2 Receptor Antagonist (**25**)	**Yes**	**Yes**	No

Summary - The pace of discovery for CRF receptor antagonists has substantially increased in the last two years as the interest in antagonists by the pharmaceutical industry has increased. As noted in the conclusions of the last report in this series (26), there are three key challenges in the area of CRF research. These include: 1) the discovery of CRF_2 subtype-selective agents to define the physiological roles of these sites; 2) the clinical evaluation of CRF_1-selective ligands and 3) the discovery of subtype-selective nonpeptide radioligands to better define the anatomical distribution of these receptors. Substantial progress has been made on all three fronts since there are now small molecule CRF_2 receptor antagonists, albeit with mixed CRF_1/CRF_2 activity, as well as results of ongoing clinical trials with selective non-peptide CRF_1 receptor antagonists that should be available in the near future.

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Chapter 3. β-Amyloid as a Target for Alzheimer's Disease Therapy

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Introduction - One of the cardinal pathological signatures of Alzheimer's disease (AD) is the deposition of the β-amyloid (Aβ) peptide in the cores of senile plaques and in the walls of cerebral blood vessels (1). This 39-42 amino acid peptide is formed from the cleavage of a larger species, the β-amyloid precursor protein (βAPP), and is prone to self-aggregation (2). It is believed that the Aβ peptide plays a key role in the pathogenesis of AD. Some evidence that supports this hypothesis is the following: (a) three familial forms of AD - trisomy 21, βAPP gene mutations, and presenilin gene mutations, *all* increase the production of Aβ; (b) the most prominent genetic risk factor for AD, which is apolipoprotein Eε4, is associated with increased Aβ deposition; (c) fibrillized Aβ is toxic to neurons *in vitro* and *in vivo*; and (d) transgenic mice overexpressing human βAPP develop AD-like cerebral amyloid plaques. One approach for blocking the formation of β-amyloid is to inhibit the polymerization of this peptide. For other amyloidoses, such as AA and AL amyloidosis, it has been shown that blocking or removing the amyloidogenic peptide, can reverse the process. Furthermore, other therapeutic targets, the β- and γ-secretases that cleave βAPP to form Aβ, have not yet been unequivocally identified. Also, Aβ aggregation inhibitors binding to the peptide may block the interactions of Aβ with other molecules involved in AD pathophysiology, including complement proteins, apolipoprotein E, heparin sulfate proteoglycan, RAGE, and scavenger receptors. The inhibition of Aβ aggregation is thus expected to slow or arrest the progress of AD, rather than treating the symptoms of the disease. This chapter will focus on the utilization of biophysical techniques for the elucidation of the actual chemical targets, highlight some of the β-amyloid inhibitors that have been reported over the past few years, and give an update on the progress of the animal models.

BIOPHYSICAL METHODS AND THE SEARCH FOR AMYLOID FIBRILLIZATION INHIBITORS

Rationale - In designing inhibitory molecules to block amyloid fibril formation, it is useful to consider the process as a protein folding problem, in which fibril formation occurs along one or more non-native pathways. The insoluble product accumulates in a relatively stable, and usually biologically disruptive state. Deriving a meaningful structure activity relationship (SAR) requires that the assay targets the rate-limiting intermediate and the compound interacts with the inhibitor in the same way, however the activity is measured. Targeting a kinetically defined, low abundance protein folding intermediate whose presence is rate-limiting, is risky. An inconsistent SAR for a protein folding reaction could be due to a change of the rate-limiting species exposing a different site, or to the existence of multiple binding modes for the inhibitors. Different inhibitors may be identified if the structure of the rate-limiting intermediates vary. Therefore, knowing details of the structures involved in the folding reactions and in the pathological amyloid fibril deposits is important in developing effective amyloid fibrillization inhibitors.

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Electron Microscopy and X-Ray Diffraction - The regularity of the organization and crossed β-sheet structure of amyloid fibrils are defined histologically by their tinctorial properties with Congo Red birefringence and Thioflavine fluorescent staining. High resolution electron micrographs of fibrils that are either negatively stained with heavy metals or shadowed with gold reveal a common structure for all fibrils, regardless of the primary sequence of the protein or peptide involved (3). Image reconstruction and cryoelectron microscopy reveal further detail in conjunction with X-ray fibril diffraction experiments, and support the existence of twisted helices of β-sheets (4-6). Even with magnetic orientation, amyloid fibrils are not sufficiently ordered to diffract to atomic resolution. X-ray diffraction studies of complexes of Congo Red with pig insulin amyloid fibrils have provided a picture of how this dye could interact with the end product of fibril formation (7-9). Inhibitors that stabilize monomeric or low oligomeric Aβ species may facilitate crystallization of these complexes.

Atomic Force Microscopy (AFM) - Kinetic studies with the Aβ peptide supporting nucleation-dependent fibril growth stimulated a search for the elusive intermediate. Electron microscopy provided some hints of small dimeric and oligomeric species that appeared early in the reaction. The smaller species gives rise to protofibrils which eventually lead to the characteristic twisted, 7 nm diameter filaments (10). Atomic force microscopy, in which a thin carbon nitride tip is dragged or tapped along a surface to reveal the topography, was adapted to biological specimens adsorbed onto the atomically smooth surface of freshly cleaved mica. Images with resolution approaching that of electron microscopy can be obtained with carbon nanotube tips (11). Fixation and staining artifacts are avoided with AFM, and the technique can be accomplished in air or under buffer solution, although only surface adsorbed material is imaged (12). A series of AFM images of Aβ(1-42) and Aβ(1-40) revealed oligomeric and protofibrillar structures that appeared to be precursors to mature amyloid fibrils (13-15). AFM and EM are complementary in characterizing the effects of different fibrillization inhibitors on the structure of reaction intermediates and in distinguishing non-specific clumping from true amyloid fibril formation.

Light Scattering - The scattering of visible light by mature amyloid fibrils (turbidity) frequently registers a lag phase since it only detects relatively large particles (≥400 nm), missing smaller, early intermediates (16). While technically simple and useful for an endpoint determination, multiple intermediate species blocked by inhibitors could remain undetected by turbidity. Inhibitors acting at different points early in the reaction would be indistinguishable. Color absorbance and precipitation of peptide and/or inhibitor further interfere with the interpretation of results.

By contrast, dynamic light scattering (quasi-elastic light scattering, QLS) measurements of scattered light intensity at multiple angles from the incident laser beam is capable of resolving nanometer size macromolecular structures at the expense of sample throughput and a requirement for specialized equipment and data analysis. It gives real-time measurements of scatterers of different sizes and is thus useful for characterizing reaction mechanisms of inhibitors identified by other methods. Small oligomeric species of Aβ have been studied by this technique (17-21). However, precipitation and inhibitor absorbance can interfere with the measurements.

Fluorescence - The extrinsic chromophore, Congo Red, and the fluorophore, Thioflavin T, have been used to monitor amyloid fibril formation for a number of amyloidogenic proteins and peptides (22-24). Some yet unelucidated property of amyloid fibril ß-sheet secondary structure elicits the spectroscopic changes, although not all ß-sheets react, particularly those in native proteins (25). Aß does not display a hydrophobic pocket detectable by 1-anilinonaphthalenesulfonic acid (1,8-ANS) binding under physiological conditions, unlike most denatured proteins. Inhibition of Thioflavin

T fluorescence has been used to search for inhibitors of amyloid fibril formation. The major caveats to this approach are compound interference with probe binding and optical artifacts. There is also the potential for false negatives for inhibitors which stabilize small, Thioflavin-positive species. Backup filtration or sedimentation assays for fibril formation can address these issues (26).

Detailed studies of the conformational state of the Aβ peptide have been hampered by the lack of intrinsic, sensitive biophysical probes. The amino acid residue substitution, Y10W, preserves fibril formation as do the substitutions, F4C or G34C, after coupling to synthetic fluorophores. Fluorescence energy transfer experiments between these probes suggest dimerization of the peptide, although unequivocal evidence is lacking (27).

Ultracentrifugation - A powerful approach to characterizing the physical state of intermediates in amyloid fibril formation is by sedimentation velocity measurements during the reaction. Early studies with Aβ provided a tantalizing look at the process, but the specialized nature of the equipment, low sensitivity, and low sample throughput restricts its utility (28). The multiple wavelength, multiple sample Beckman XLA ultracentrifugation system, has made these experiments accessible. Species of all molecular sizes can be followed down to the low micromolar range as long as the species that is being monitored has a relative abundance of 5-10% and the species are stable during the centrifugation run. The formation of amyloid fibrils from transthyretin has been characterized by this approach (29).

Nuclear Magnetic Resonance (NMR) - NMR can be used to determine the solution and solid state conformations of peptides and their state of oligomerization, as well as their association with small molecules. Stringent depolymerization of Aβ(1-40) and Aβ(1-42) preparations yields a metastable monomeric species at physiological pH, low ionic strength, and low temperature, which has been used to study interactions of the monomer with nicotine and melatonin (30-32). Solution phase structure determination depends, in a complex way, upon the exchange rates of inhibitor binding and the rates of structural interconversion of peptide conformations. Solid state NMR has been used to characterize the amyloid fibril structure formed by the Aβ(34-42) fragment (33). Methodological improvements promise to extend the utility of the solid state method to smaller amounts of longer peptides and to the binding of small molecules.

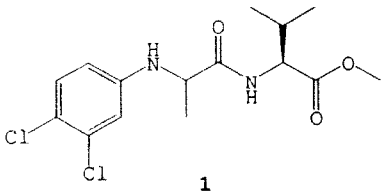
Fourier-Transform Infrared Spectroscopy (FTIR) - FTIR has been used to garner secondary structure information for both soluble and fibrillar Aβ peptides. ^{13}C-Isotope editing of the synthetic C-terminal fragment, Aβ(34-42), localized an abnormal conformational state in the Gly37, Gly38 region (34). Availability of recombinant, selectively isotope-enriched peptides and/or small molecule binders could lead to a resurgence in these types of studies (35). The protein in senile plaques of Alzheimer's Disease brain tissue has been shown by FTIR microscopy to be mostly in an amyloid-like crossed β-sheet conformation (36).

β-AMYLOID INHIBITORS

Secretase Inhibitors - Much of the current research is directed towards the design of inhibitors that block the yet unidentified enzymes, α-, β-, and γ-secretases, that cleave the β-amyloid precursor protein (βAPP) to form Aβ. Inhibition of the β- and γ-secretases should reduce Aβ formation and thus slow the progression of Alzheimer's

Disease. Sequential proteolysis liberates Aβ from βAPP, i.e., β-secretase cleaves at the N-terminus of Aβ, followed by C-terminal γ-secretase hydrolysis (37-39).

Amino acid derivatives, **1** and **2**, inhibit the release and/or synthesis of Aβ, and therefore, are claimed as being useful in the prevention and treatment of Alzheimer's (40, 41).



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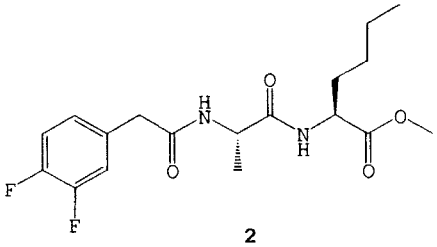
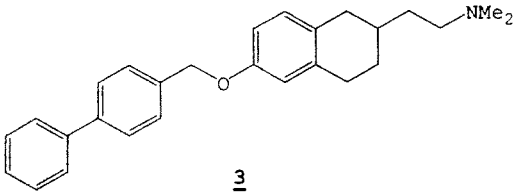


2

Amine and urea analogs, **3** and **4**, have also been claimed in the patent literature as Aβ production inhibitors (42, 43). It was reported that **3** inhibits production and/or secretion of Aβ1-40 and Aβ1-42 by 74% and 75%, respectively, relative to control, whereas **4** inhibits Aβ production activity in cells at 10μg/mL (IC_{50}=2.8μM).



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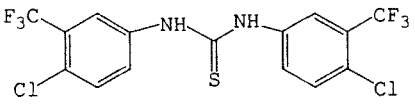
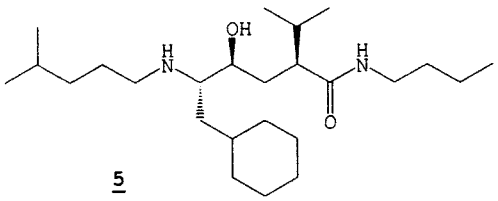


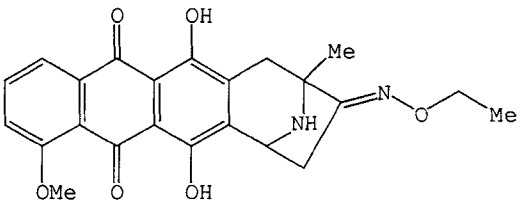
4

Most of the compounds reported are claimed for the inhibition of β-amyloid protein production rather than as secretase inhibitors. Another such series of compounds are hydroxy-hexanamide derivatives, such as **5**, which are expected to be effective in treating patients suffering from the cerebral accumulation of β-amyloid protein, such as in Alzheimer's Disease (44).



5

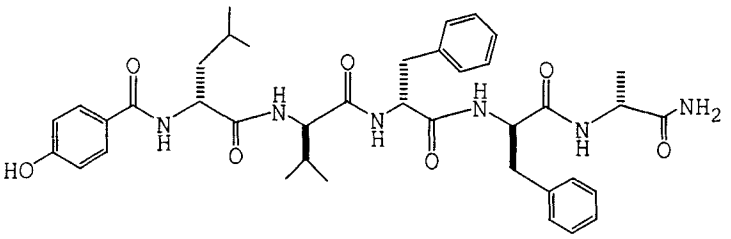
Aggregation Inhibitors – Aβ is postulated to be a key player in the series of events culminating in neurodegeneration and clinical dementia (1). Previously reported antiaggregation inhibitors such as IDOX and Rifampicin are being improved (45). Imino-aza-anthracyclinone derivatives of IDOX, such as **6**, have recently been claimed as useful for the prevention of amyloid formation and/or the induction of amyloid degradation in peripheral and CNS amyloidoses (46).



6

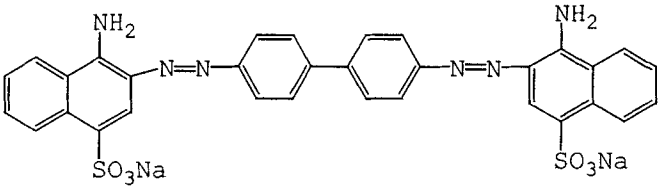
Other high molecular weight molecules, such as Amphotericin B, porphyrins and phthalocyanines have also been reported to have anti-aggregation effects (47, 48).

Short, modified peptides derived from the Aβ sequence, such as 7, have been shown in some assays to prevent fibril formation and Aβ toxicity (49).



7

Small molecule inhibitors from various chemical classes have recently been reported and reviewed (50, 51). These compounds inhibit or reduce *in vitro* aggregation of Aβ peptides, although the peptide species being inhibited is unknown. Somewhat similar in structure to Congo Red 8, the amyloid staining agent that inhibits the formation of Aβ(1-40) fibrils, are naphthyl monoazo derivatives, such as 9 (52). These types of molecules are claimed for imaging amyloid and blocking amyloid deposition (53).



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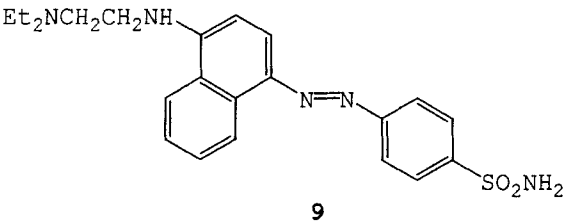
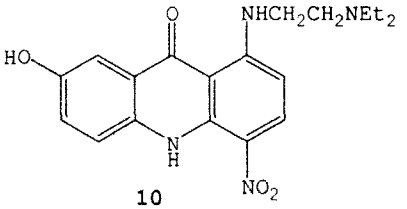


9

Other molecules with similar properties towards β-amyloid are acridinones and benzothiazole derivatives, such as **10** and **11** (54, 55).



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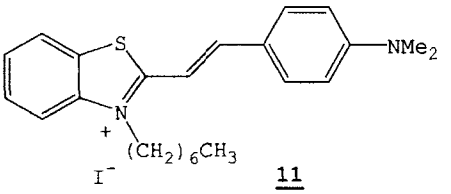
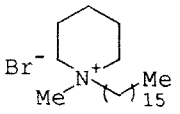


11

Quaternary N-alkyl ammonium salts, such as hexadecyl-N-methylpiperidinium bromide **12** (HMP), form stoichiometric complexes with the Aβ-peptide well below their critical micellar concentration, suggesting that these types of compounds are not acting solely as detergents (56). Simple disulfonates and disulfates, such as 1,4-butanedisulfonic acid disodium salt **13**, interfere with splenic amyloid deposition in a mouse model of AA amyloidosis (57).



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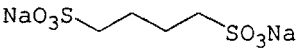
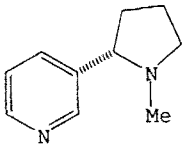


13

Finally, nicotine **14** and melatonin **15** and some of their derivatives have been reported to be effective at high concentrations in preventing Aβ fibril formation under specified conditions (58, 59). From NMR studies and the apparent stoichiometry, these molecules appear to interact primarily with the monomeric peptide. This is the only instance in which the nature of the peptide that is interacting with an inhibitor is known.



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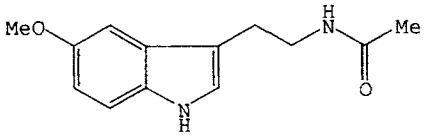

15

INHIBITION OF Aβ TOXICITY

One of the ephemeral properties of the Aβ peptide is its apparent toxicity in some cellular and animal models (60-62). The irreproducibility among different laboratories and between different lots of various synthetic Aβ peptides, depending on its past history of the peptide is matched only by the different toxicity assays used and the variety of cellular systems affected. A particular conformational state of the peptide seems to be required and a low abundance non-fibril intermediate species may be responsible for the peptide's effects, possibly through a surface receptor(s) (63-67). While toxicity has been a popular target, the physiological response can be

ameliorated by so many things that increase cellular tolerance to insults that the specificity needed to develop an inhibitor SAR becomes difficult to achieve (68). Secretase inhibitors, antioxidants, aggregation inhibitors, and metabolic enhancers could all diminish Aβ toxicity. Aβ-specific central nervous system animal models that demonstrate toxicity are not readily available.

ANIMAL MODELS

Testing inhibitors of β-amyloid production or deposition ultimately requires a suitable animal model. Fortunately, with the advent of transgenic mice bearing various forms of the human βAPP gene, a number of animals are now available. There are also species that develop β-amyloid deposits naturally with age, particularly dogs and nonhuman primates (69-74). Because of their relatively large brains, these animals are particularly well-suited to the development of Aβ-imaging strategies, but their long life-spans and genetic heterogeneity are drawbacks for testing therapies. Transgenic mice, on the other hand, can develop impressive quantities of cerebral β-amyloid deposits, in some cases beginning as early as four months of age.

A number of βAPP transgenic mouse lines have now been produced (75). Three well-characterized models that develop ample cerebral amyloid are the Athena (PDAPP) mouse, the Hsiao (Tg 2576) mouse, and the Novartis (APP23) mouse (76-78). The characteristics, advantages and disadvantages of the various transgenic models of AD have recently been thoroughly reviewed (75). Currently, only the Tg 2576 model is widely available to academic and industrial researchers. This model will be described in more detail, as well as the results of crossed βAPP and presenilin 1-transgenics. It should be borne in mind that the existing transgenic models have a number of interesting and useful characteristics, but none yet fully recapitulates the behavioral and pathological features of AD.

The Hsiao Tg 2576 mice express the human βAPP transgene, with the "Swedish" double mutation K670N-M671L (77). The transgene is driven by a hamster prion protein promoter, and expression is primarily in neurons, but also occurs to a lesser degree in astrocytes. The expression of the transgene is approximately five to six times endogenous mouse βAPP, and remains stable throughout adulthood. Around the age of nine to ten months, Aβ levels in the brain start to rise markedly, and the Tg 2576 mice begin to deposit Aβ in diffuse and compact senile plaques. Occasionally, cerebral blood vessels also are amyloidotic (74). The neocortex and hippocampus are the most affected regions. By sixteen months of age, amyloid deposition can be copious, although variability among animals is high (79). The amyloid that is deposited in the neuroparenchyma is in the form of diffuse and compact senile plaques. In many ways, these plaques resemble those seen in humans with AD. The compact deposits are birefringent after staining with Congo red, are surrounded by abnormal neurites and astrocytes, and are closely associated with microglial cells (80). Homozygous Hsiao mice have not yet been produced. At present, these mice must be maintained on a hybrid background (C57Bl6/SJL), or they will not survive long enough to develop senile plaques. Recently, it has been shown that Hsiao mice crossed with mutant presenilin-1 (PS1M146L) transgenic mice have accelerated deposition of amyloid (81, 82). This observation has also been made with a different line of mutant βAPP/PS1 transgenics (83). These findings indicate a significant interaction of presenilin 1 and Aβ *in vivo*, and provide animal models with more rapid, and possibly less variable, Aβ deposition than in uncrossed βAPP transgenics.

The arrival of transgenic models of cerebral β-amyloidosis is a boon to the AD therapeutic research community. The small size and short life span of these animals make feasible the relatively rapid and inexpensive testing of inhibitors of Aβ

production and aggregation. Imaging methodology may soon improve to the point where *in vivo* imaging of amyloid load in mice is also possible. Meanwhile, such studies can be profitably undertaken in larger species. A key question that remains to be answered is whether compounds that inhibit Aβ production or aggregation in animal models will impede the pathogenesis of AD. This issue and others regarding the relationship of transgenic models to the human disease should soon be resolved.

Conclusion – Over the past few years, there has been significant progress in the field of Alzheimer's disease, especially in the areas of biophysical techniques to assist with the elucidation of the actual chemical targets, and in the development of the transgenic mouse models. Continued efforts in these areas, as well as towards the identification of potential chemical inhibitors, should assist in bringing forth potential drug candidates for clinical evaluation in the AD arena.

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Chapter 4. Recent Advances in the Development of Neuropeptide Y Receptor Antagonists

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Introduction - Neuropeptide Y (NPY) is a 36 amino acid neuropeptide that is widely distributed in both the central and peripheral nervous systems (1). NPY is a member of the pancreatic polypeptide family that also includes the structurally related peptides peptide YY (PYY) and pancreatic polypeptide (PP) (1). As expected from its ubiquitous distribution, NPY has been implicated in a wide variety of physiological effects and, hence, has become a target of great interest to many pharmaceutical companies. In this chapter, recent advances in our understanding of the biology of NPY will be discussed and progress toward the development of selective NPY receptor ligands will be reviewed.

NPY RECEPTORS

NPY and other members of the pancreatic polypeptide family elicit their physiological effects via interaction with at least six distinct G protein-coupled receptors designated Y_1, Y_2, Y_3, Y_4, Y_5 and y_6 (2,3). A PYY-preferring receptor has also been postulated to exist, but the existence of this receptor is controversial (2,3). In contrast to other families of G protein-coupled receptors, the NPY receptors share only modest primary sequence homology (30-50%) (2,3). The structural differences among NPY receptors is beneficial to drug discovery efforts since compounds with high affinity for a particular NPY receptor are less likely to interact with other NPY receptors. In addition to having distinct protein sequences, each of the NPY receptors is characterized by a unique pharmacological profile and a distinct tissue localization (2,3).

Y_1 Receptor - The NPY Y_1 receptor is pharmacologically characterized by its high affinity for [Pro^{34}]NPY and its low affinity for PP and for N-terminally truncated NPY analogues (2,3). Activation of this receptor decreases cAMP formation, increases intracellular calcium levels and increases MAP kinase activity (4,5). The Y_1 receptor is localized in many brain regions and in the vascular smooth muscle of most blood vessels (2,3).

Y_2 Receptor - The NPY Y_2 receptor is pharmacologically unique in that it has high affinity for N-terminally truncated NPY analogues, but has low affinity for [Pro^{34}]NPY (2,3). Like the Y_1 receptor, activation of the Y_2 receptor decreases cAMP formation, increases intracellular calcium concentration and increases MAP kinase activity (5,6). The Y_2 receptor is localized in several brain regions and on the terminals of peripheral sympathetic neurons (2,6).

Y_3 Receptor - The NPY Y_3 receptor is pharmacologically characterized by its lower affinity for PYY than for NPY (2,3). Activation of the Y_3 receptor decreases cAMP levels and increases calcium influx (2,7). The Y_3 receptor has been found in rat heart, colon, adrenal gland, brainstem and hippocampus (2,3).

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Y_4 Receptor - The NPY Y_4 receptor has very high affinity for PP and much lower affinity for NPY (2,3). Therefore, the Y_4 receptor is probably a PP receptor rather than an NPY receptor. Activation of the Y_4 receptor decreases cAMP formation and increases intracellular calcium concentration (2). The Y_4 receptor is sparsely expressed in the brain (2). In the periphery, the Y_4 receptor is expressed primarily in the rat testis and lung (2). A different pattern of expression is seen in man, with expression primarily detected in colon, small intestine, prostate and pancreas (2).

Y_5 Receptor - The NPY Y_5 receptor is pharmacologically distinguished by its high affinity for both N-terminally truncated analogues of NPY and [Pro^{34}]NPY (2,3). In addition, the NPY Y_5 receptor has high affinity for human PP, but much lower affinity for rat PP (2,3,8). Activation of the NPY Y_5 receptor decreases cAMP formation and increases intracellular calcium concentration (8,9). The NPY Y_5 receptor is localized primarily in the brain, but is also detected in testis, spleen and pancreas (8,10).

y_6 Receptor - The NPY y_6 receptor has a pharmacological profile that is similar to the NPY Y_1 receptor, but is somewhat more tolerant of N-terminal truncation (2,3). A functional NPY y_6 receptor gene is found in mice and rabbits, but the NPY y_6 receptor gene is a nonfunctional pseudogene in primates (2,11). An NPY y_6 receptor gene cannot be detected in rats (11). Activation of the NPY y_6 receptor decreases cAMP formation (11). In mice, expression of the NPY y_6 receptor has been detected in the kidney, testis and brain, particularly in the hypothalamus (2,12).

PHYSIOLOGICAL ROLES OF NPY

Body Weight - Several lines of evidence suggest that NPY plays a key role in the control of body weight. Central administration of NPY dramatically increases food intake and reduces energy expenditure in satiated animals, while reduction of endogenous NPY levels via immunoneutralization or antisense oligonucleotide techniques leads to a decrease in food intake (for references, see 13). As would be expected for an orexigenic peptide, hypothalamic NPY peptide and mRNA levels are increased after fasting and in genetically obese rodents (for references, see 13). Furthermore, NPY-containing neurons either co-express other neuropeptides that are thought to be involved in the regulation of body weight or interact with other neuronal populations that express such neuropeptides (14).

Despite the evidence that NPY is involved in the regulation of body weight, mice lacking NPY have normal food intake and body weight under a wide variety of conditions (15). This may indicate that NPY is not a critical component of the systems that regulate body weight. Alternatively, mice may be able to compensate for the lack of NPY or NPY may act only in very specific physiological situations. In support of the latter possibility, a cross between the NPY-deficient mouse and the leptin-deficient *ob/ob* mouse demonstrates that NPY contributes significantly to the obesity, endocrine abnormalities and reproductive abnormalities of the *ob/ob* mouse (16). Thus, NPY may be a critical regulator of body weight under conditions where leptin levels are very low (e.g. during dieting or other conditions of prolonged, marked weight loss).

Both the NPY Y_1 and Y_5 receptors appear to mediate the effects of NPY on food intake (17). Administration of NPY Y_1 or Y_5 receptor antagonists partially decreases spontaneous and NPY-induced food intake (9,18). Similarly, mice lacking the NPY Y_1 or Y_5 receptors have a partial reduction of NPY-induced food intake (19,20). Mice

lacking the NPY Y_5 receptor have normal food intake and body weight under a wide variety of conditions (19), whereas mice lacking the NPY Y_1 receptor have slightly decreased spontaneous food intake and significantly decreased deprivation-induced food intake relative to control mice (20). While these data support a role of the NPY Y_1 receptor, but not the NPY Y_5 receptor, in the regulation of body weight, further experiments are needed to fully understand the phenotype of the knockout mice. Overall, the data suggest that NPY Y_1 and/or NPY Y_5 receptor antagonists might be useful anti-obesity agents.

Anxiety - Central administration of NPY is anxiolytic in a number of behavioral paradigms (21). Pharmacological studies and antisense oligonucleotide studies suggest that the anxiolytic effect of NPY is mediated by the NPY Y_1 receptor (21,22). Therefore, NPY Y_1 receptor agonists might represent novel anti-anxiety agents.

Neuronal Excitability/Seizure Activity - NPY mRNA and protein levels are increased by experimentally-induced seizures and NPY has been shown to decrease epileptiform discharges elicited by a variety of stimuli *in vitro* and *in vivo* (23). In addition, mice lacking NPY have a relatively high incidence of spontaneous seizures and are more sensitive to agents that induce seizures (15). These data suggest that NPY is an endogenous anti-epileptic agent and that NPY receptor agonists might be useful anti-epileptic drugs. While the NPY Y_2 receptor has been thought to mediate the effects of NPY on neuronal excitability *in vitro* (23), activation of the NPY Y_5 receptor has been claimed to inhibit kainic acid-induced seizure activity *in vivo* (24).

Cardiovascular Function - NPY contracts vascular smooth muscle *in vitro* and exerts hypertensive effects *in vivo*, particularly under conditions of high sympathetic nervous system activity such as stress (25). The pressor effects of NPY *in vivo* and the contractile effects of NPY on vascular smooth muscle *in vitro* are mediated by the NPY Y_1 receptor (20,26). Application of NPY to the brainstem has also been shown to decrease blood pressure and heart rate, effects that may be mediated by the NPY Y_3 receptor (2).

Recently, a genetic association between a nucleotide sequence polymorphism in the signal peptide of NPY and high plasma cholesterol levels was reported in Finnish and Dutch populations (27). Additional studies will be required to confirm this association and to determine the mechanism by which NPY may modulate plasma cholesterol levels.

Ethanol Consumption - Rats that have a predisposition to consume large amounts of alcohol have been shown to have lower levels of NPY in several brain regions than rats that do not choose to consume alcohol (28). As predicted by these findings, mice lacking NPY were recently shown to consume significantly more alcohol and were less sensitive to the sedative/hypnotic effects of alcohol than normal mice (29). If NPY plays a similar role in determining alcohol preference and sensitivity in man, then NPY receptor agonists may find use in the treatment of alcohol addiction. However, the NPY receptor(s) mediating this effect of NPY is not known.

Endocrine Function - NPY regulates the secretion of various pituitary hormones, including growth hormone, gonadotropins and adrenocorticotrophic hormone (ACTH) (2,30) and, hence, affects reproductive and endocrine function. The NPY receptor that mediates the effect of NPY on pituitary hormone secretion is unclear. The

receptor mediating the stimulation of pituitary ACTH secretion has been pharmacologically characterized and appears to be most closely related to the NPY Y_5 receptor (30).

Systemic administration of NPY has been reported to decrease blood glucose, increase glucose utilization and inhibit insulin secretion (31,32). The ability of NPY to decrease plasma glucose has been claimed to be mediated by activation of the NPY Y_5 receptor (31), but experiments with selective agonists and/or antagonists are required to confirm this claim.

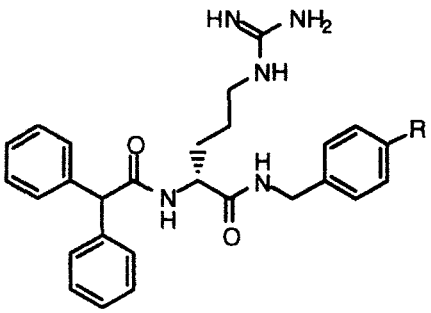
NPY RECEPTOR ANTAGONISTS

Y_1 Receptor Antagonists - The dimeric peptide **1** (1229U91) is a potent, rationally designed Y_1 receptor antagonist (human Y_1 $pA_2 = 10.5$, rat Y_1 $pA_2 = 10.0$), but is also a potent agonist at human Y_4 and mouse y_6 receptors (pEC_{50} = 8.6 and 9.1, respectively) (33,34,35). **1** is a weak agonist at human Y_2 and Y_5 receptors (34,35). **1** has been shown to inhibit spontaneous, NPY-stimulated and fasting-induced food intake in lean rats and in Zucker rats (36,37). In Zucker rats, **1** did not affect galanin-stimulated food intake, indicating that the effects of **1** on food intake were NPY-specific (37). Although these studies support a role of the Y_1 receptor in feeding, the utility of **1** as a tool for elucidating the physiological role of the Y_1 receptor is compromised by its lack of selectivity.

Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-NH_2
| |
H_2N-Tyr-Arg-Leu-Arg-Tyr-Dpr-Pro-Glu-Ile

1 1229U91

The first potent and selective nonpeptide NPY Y_1 receptor antagonist was **2** (BIBP3226) (38). A more potent analogue, **3** (BIBO3304), was subsequently described (18). Compounds **2** and **3** have high affinity for the NPY Y_1 receptor (rat Y_1 K_i = 6.8 nM and rat Y_1 IC_{50} = 0.72 nM, respectively) and are inactive at the other NPY receptor subtypes (K_i > 1,000 nM) (8,18). Compounds **2** and **3** also block NPY-induced inhibition of forskolin-stimulated cAMP synthesis in cells expressing the NPY Y_1 receptor (pK_b = 8.2 and 9.1, respectively), confirming their antagonist character. Compound **2** blocks NPY-induced contraction of various vascular smooth muscle preparations *in vitro* and also blocks the pressor response to NPY in pithed rats (26). Although **2** had no effect on basal blood pressure, the compound attenuated the pressor response induced by cold stress in rats (26). These data suggest that NPY acts via the Y_1 receptor to induce vasoconstriction during stress, but does not influence vascular tone under normal conditions.



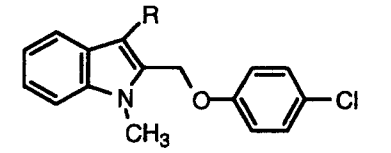
2 R = -OH BIBP3226

3 R = -CH_2HN-C(=NH)-NH_2 BIBO3304

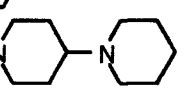
Compounds **2** and **3** do not penetrate the blood-brain barrier when administered systemically (26) and, hence, must be administered centrally to investigate the role of Y_1 receptors in the CNS. Administration of **2** into the paraventricular nucleus of rats was reported to block NPY-induced food intake (39,40). However, observations of barrel-rolling and catatonia seen on central administration of **2** have cast doubt on the specificity of these effects (26, 41). Compound **3** is claimed to cause less CNS toxicity and, hence, more recent studies have examined the effects of this compound on feeding behavior (18). Centrally administered **3** to rats was reported to attenuate NPY- and deprivation-induced feeding (18). There was no effect of **3** on feeding induced by noradrenaline or galanin and the inactive enantiomer of **3** (Y_1 K_i > 1,000 nM) did not inhibit NPY- or deprivation-induced feeding, suggesting that the effects of **3** on food intake were specifically due to Y_1 receptor antagonism. In addition, **3** delayed body weight gain when administered icv for one week to Zucker and Wistar rats (42). These data indicate that activation of the Y_1 receptor normally promotes food intake and body weight gain, but the possibility that other NPY receptor subtypes are involved in the regulation of body weight is not precluded.

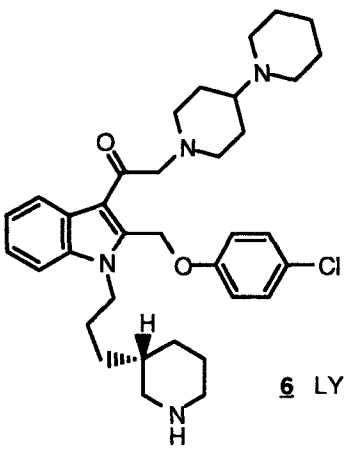
Central administration of **2** to rats has been reported to elicit anxiogenic effects (43). This is consistent with an anxiolytic role of NPY mediated by Y_1 receptors and suggests that anxiogenesis could be a potential side effect of Y_1 receptor antagonists.

A series of trisubstituted indole NPY Y_1 receptor antagonists have been developed from lead compound **4** (human Y_1 K_i = 2.1 μM), which was discovered through biased library screening (44). Structural modifications at C3 resulted in the homologated carbonyl derivative **5** (human Y_1 K_i = 26 nM). The improved potency of **5** was ascribed to the additional basic substituent and a favorable spatial orientation conferred by the C3 carbonyl. Incorporation of an additional basic group at N1 afforded **6** (LY 357897). Compound **6** displayed a K_i of 0.75 nM at the cloned human Y_1 receptor, and K_i's of > 10 μM at cloned human Y_2, Y_4, and Y_5 receptors. The compound was found to antagonize NPY-induced inhibition of forskolin-stimulated cAMP (K_i = 1.8 nM) and NPY-induced Ca^{2+} mobilization (K_i = 3.2 nM) in cells expressing the human Y_1 receptor. When co-administered with NPY (0.23 nmol) into the lateral ventricle of mice, **6** dose-dependently blocked NPY-induced food



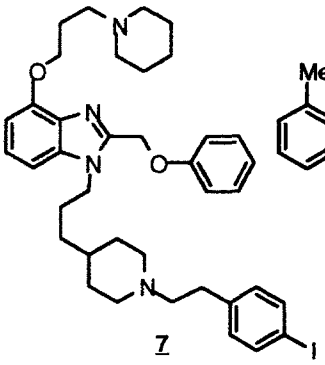
4 R = $-H_2C-$

5 R = $-OC-H_2C-$ 



6 LY 357897

consumption with an ED_{50} of 17 nmol. However, 6 could not be evaluated systemically due to a lack of oral and subcutaneous bioavailability (44). Related series of benzimidazole and benzothiophene Y_1 receptor antagonist are exemplified by 7 - 9 which were reported to display Ki's at human Y_1 receptors of 0.052 nM, 4.5 nM, and 11 nM, respectively (45-48). No additional biological data for these compounds was reported.



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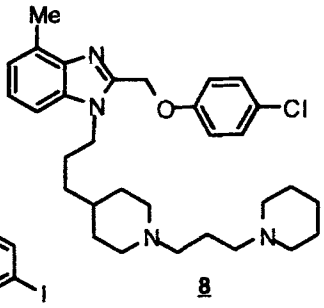

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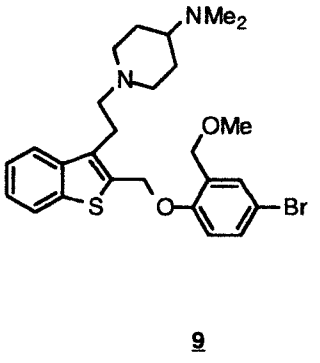

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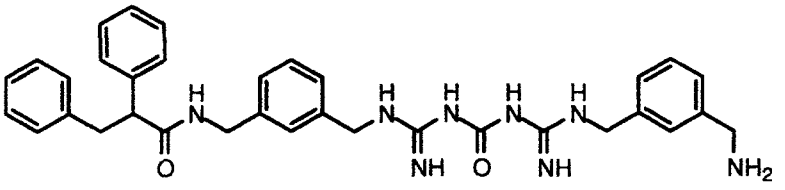
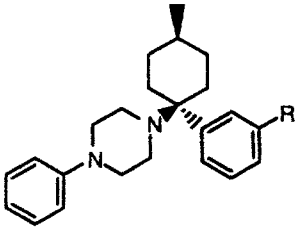
The bis-amidinourea 10 was reported to be a Y_1 receptor antagonist with an IC_{50} of 50 nM at the human Y_1 receptor (49,50). The selectivity of 10 at other NPY receptor subtypes was not disclosed. The orexigenic effect of NPY in rats was reported to be blocked on co-administration of 10 (50). Further studies to address the specificity of the effects of 10 on food intake are required.



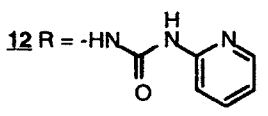
10

Benzylamine derivatives represented by compound 11 were previously disclosed in a patent application (51). Compound 11 was reported to inhibit [^{125}I]PYY binding to human Y_1 receptors with an IC_{50} of 67 nM. More recent patent applications describe compounds such as 12 - 14 that feature expanded aryl substitution (52-54). No biological data for these compounds was reported.

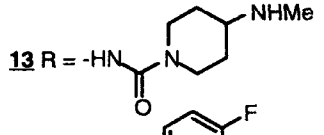


11 R = -OMe

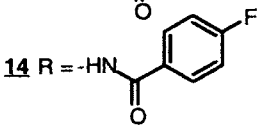
12 R = -HN



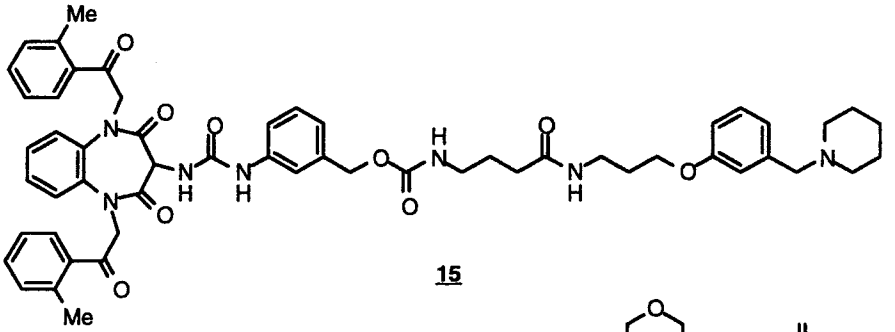
13 R = -HN



14 R = -HN



The benzodiazepine 15 (Y_1 K_i = 6.4 nM) is a novel Y_1 receptor antagonist that was discovered by random screening (55). Compound 15 did not show significant affinity for Y2 and Y5 receptors (IC_{50}'s > 1 μM), but was reported to have affinity for cholecystokinin B receptors (IC_{50} = 19 nM) and histamine H_2 receptors (pA_2 = 5.8) (55). A related series of benzazepinone Y_1 receptor antagonists of lower molecular weight represented by 16 (Y_1 IC_{50} = 3.2 nM) has been disclosed in the patent literature (56). The diaminopyridine 17 (Y_1 IC_{50} = 0.33 nM), also reported in the patent literature, is representative of an additional class of Y_1 receptor antagonists (57).



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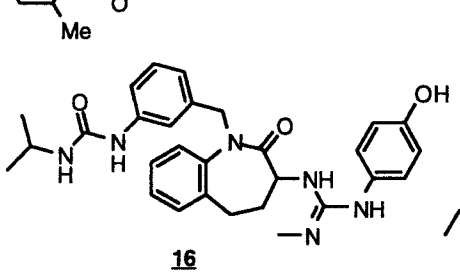

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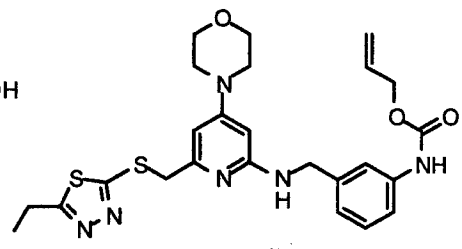
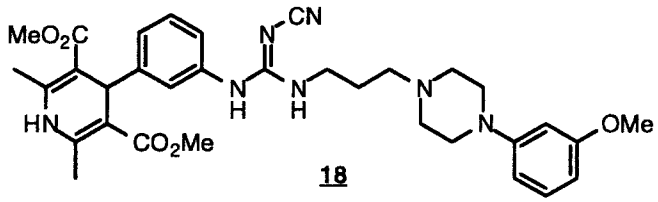


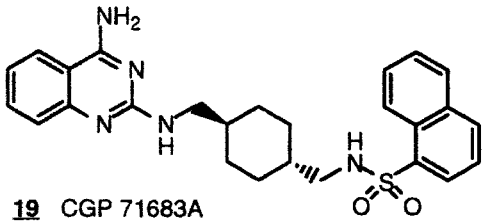
17

Further patent applications claim dihydropyridine and dihydropyrimidone derivatives, exemplified by 18, as Y1 receptor antagonists. No specific biological data for these compounds was reported (58-60).



18

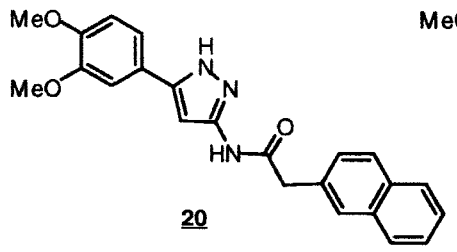
Y_5 Receptor Antagonists - A series of quinazoline Y_5 receptor antagonists has recently been disclosed in the patent literature (61-64). Pharmacological characterization of one compound in this class, 19 (CGP 71683A), has been reported (9). Compound 19 has high affinity for the rat NPY Y_5 receptor (IC_{50} = 1.4 nM), but has low affinity for the other NPY receptor subtypes (IC_{50} > 1,000 nM). The compound was an antagonist of the rat Y_5 receptor, selectively inhibiting NPY-induced Ca^{2+} transients. In satiated lean rats, intraperitoneal injection of 19 blocked NPY-induced food intake by 50%, but had little effect on spontaneous food intake. Rats that were chronically dosed with 19 showed dramatically reduced food intake



19 CGP 71683A

and body weight gain over the first 3 days of treatment, but food intake and body weight gain subsequently approached control levels. Animals that had received 19 displayed significantly decreased fat pad weights relative to pair-fed animals. Whether the reduced fat pad weights was a consequence of non-specific effects caused by high local concentrations of 19 near the injection site, or a consequence of blockade of central Y_5 receptors (e.g. a thermogenic effect or increased lipoprotein lipase activity) is not clear (9). Development of orally bioavailable selective Y_5 receptor antagonists is required to address this issue.

Several structurally distinct small molecule Y_5 antagonists have recently been claimed in the patent literature for the treatment of obesity and related disorders. A series of patent applications disclosed aminopyrazole derivatives represented by 20 and 21, with reported IC_{50}'s at human Y_5 receptors of 8.3 nM and 27 nM respectively (65-68). No *in vivo* data has been reported for these compounds.



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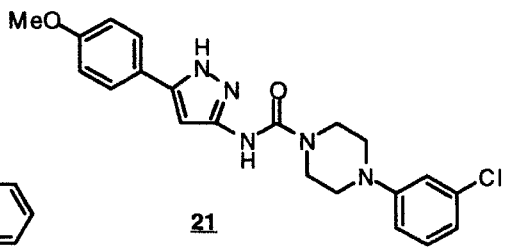

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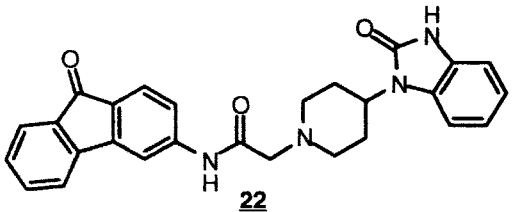

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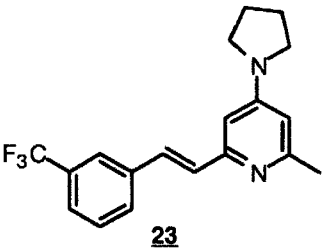

23

Additional patent applications claim amide derivatives represented by 22 as selective Y_5 receptor antagonists (69,70). Compound 22 displayed an IC_{50} at human Y_5 receptors of 0.47 nM, with no significant affinity for human Y_1, Y_2, and Y_4 receptors (69). The aminopyridine derivative 23 (human Y_5 IC_{50} = 4.2 nM) represents another class of Y_5 receptor antagonists (71). No *in vivo* data for these compounds has been reported.

Conclusion - Given the physiological importance of NPY, there is much interest in the development of therapeutic agents that modulate NPY function under pathophysiological conditions. Significant progress has been made in the development of selective Y_1 receptor antagonists, and reports of selective Y_5 receptor antagonists have begun to emerge. These agents, as well as antagonists

with improved pharmacokinetic properties, will clarify the pathophysiological role of NPY in obesity and related disorders. Such agents will also allow an assessment of the potential for Y_1 and Y_5 receptor antagonists to ameliorate these conditions. Finally, there is a clear need to identify potent and selective antagonists of Y_2 and Y_4 receptors to further clarify the relevance of NPY in disease.

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Chapter 5. Pharmacological Interventions in the Sleep Process

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Introduction – Insomnia is the term used to describe difficulties initiating sleep, maintaining sleep, or obtaining a restorative sleep. It is a symptom that can arise for a multitude of reasons, but it is clear that it represents a significant public health problem. A report from a symposium held by the World Health Organization indicates that about one-third of the adult population of the US experiences insomnia and that for about 10% it is a serious or chronic problem (1). About 40% of these individuals will attempt to self-medicate with over-the-counter agents or alcohol, indicating that a clear need still exists for accepted, effective medications. Since the likelihood of severe or chronic insomnia increases with age, the management of insomnia in the geriatric population is also a significant concern (2,3). This chapter will overview current research aimed at improved pharmacological intervention in the sleep process.

$GABA_A$ Benzodiazepine Receptor Ligands - The benzodiazepines (BZs), which were introduced in the 1960's, remain the mainstay of hypnotic therapy. It is generally accepted that the BZs produce their effects by acting at benzodiazepine receptors (BZRs) which are allosteric modulatory sites of the $GABA_A$ receptor. There is a tremendous amount of evidence to support a role for $GABA_A$ receptors in the regulation of sleep as compounds that interact with $GABA_A$ receptors produce pronounced effects on sleep-wake behavior. The effects of peripherally administered $GABA_A$ modulators on sleep have recently been reviewed (4).

$GABA_A$ receptors are a heterogeneous family of fast-acting ligand-gated anion channels which are primarily responsible for mediating the depressant actions of GABA, the major inhibitory neurotransmitter in the mammalian CNS. The $GABA_A$ receptor complex appears to be a pentamer composed of combinations of structurally related submit families (α, β, γ, δ and ρ) some of which exist in multiple isoforms (5). A channel with the properties of native receptors requires the combination of at least one α, one β and one γ-subunit with the precise combination of protein subunits determining the physiologic and pharmacologic properties of a $GABA_A$ receptor. It is the α-subunit that bears the BZ binding site (6). The BZ type 1 receptor, which displays high affinity for a number of BZs and the imidazopyridine zolpidem, contains an α1 subunit whereas the BZ type II receptor, which has a lower affinity for these agents, contains α2 or α3-subunits. Since many compounds with affinity for BZRs belong to non-BZ chemical classes, it is less confusing to refer to these receptors by their alternate nomenclature, ω_1 for BZ type 1 and ω_2 for BZ type 2 (7).

Although the BZs have proven efficacy in insomnia and are generally considered to be safe, their use is associated with a number of adverse effects including residual daytime sedation (hangover), rebound insomnia, anterograde amnesia and, upon chronic administration, tolerance and dependence (8). Newer agents have sought to overcome some of these side-effects by selectively targeting ω_1 receptors that are located in areas of the brain that are involved in sedation vs. ω_2 receptors which are highly concentrated in areas responsible for cognition, memory and psychomotor

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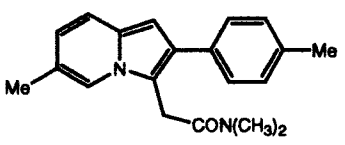
functioning. Work in this area has led to the development of effective agents that are structurally unrelated to the BZs.

Zolpidem (1) was the first non-BZ ω_1 agonist to be marketed in the U.S. It selectively binds to ω_1 receptor subtypes and possesses minimal anticonvulsant, myorelaxant and anxiolytic properties at hypnotic doses (9). The compound has a rapid onset of action and a short elimination half-life (1.5 – 2.4 h). Unlike the BZs, it improves sleep latency and total sleep time without adversely altering physiological sleep architecture. The use of zolpidem does not appear to produce rebound insomnia or impair daytime performance (10).



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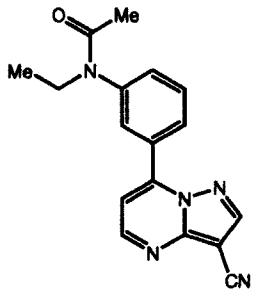

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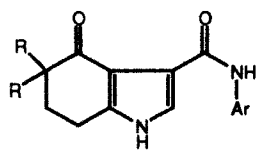
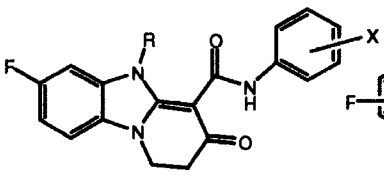
The newest non-BZ ω_1 agonist on the market is zaleplon (2). This compound recently received approval in the European union as a treatment for insomnia and it has been deemed "approvable" in the US by the FDA (11). Like zolpidem, zaleplon binds preferentially to ω_1 receptors and produces sedative effects (12). However, its affinity for the site labeled by ^{3}H-flunitrazepam is weaker than that of zolpidem suggesting a faster receptor off-rate and perhaps a better therapeutic ratio. Zaleplon is characterized by very rapid absorption and elimination ($T_{1/2}$ in plasma and T_{max} = 1h) which leads to a very fast onset of activity, a very short duration of action and a lack of next day residual effects.

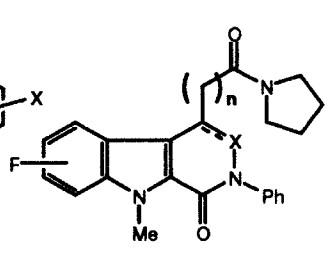
Interest in non-BZ modulators for $GABA_A$ receptors remains high as evidenced by the continuing patent activity in this area. It is beyond the scope of this chapter to provide an extensive update to previous reviews of active chemotypes (13,14). However, it is worth noting several chemotypes that stand out in the recent patent literature due to the publication of multiple, closely related patent applications. An extensive series of $GABA_A$-selective fused pyrrolecarboxamides exemplified by 3 and 4 are reported to displace ^{3}H-flumazenil from rat cortical membranes (K_i = 2 nM and 0.8 nM, respectively) with high affinity (15-20). Work continues in a series of 3-oxo-pyrido(1,2-a)benzimidazole-4-carboxamides (5,6) that possess potent affinity for $GABA_A$ receptors as determined by ^{3}H-flunitrazepam (IC_{50}'s = 0.096 and 0.072 nM, respectively) binding assays (21,22). Pyrido[3,4-b]indole-4-carboxamides (7) and pyridazino[4,5-b]-indole-1-acetamides (8) are being generically claimed as ligands for ω_1 and ω_2 receptors (23,24).



3 R = Me Ar = 2-thiazolyl
4 R = H Ar = 2-OH-phenyl



5 R = 3-thienylethyl X = 3-F
6 R = CH_2CN X = 2-F

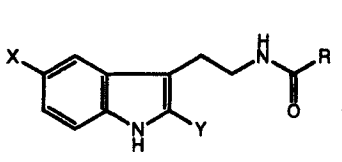


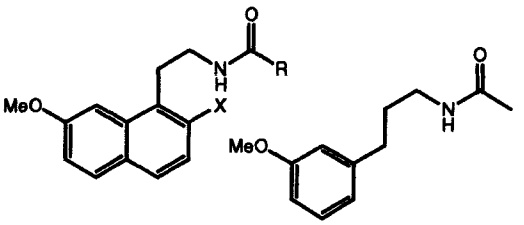
7 X = C n = 0
8 X = N n = 1

Melatonin Receptor Ligands – The daily sleep-wake cycle is the most easily recognized circadian rhythm in man and it is possible that disrupted sleep is a consequence of abnormalities in the mechanisms that control this circadian rhythmicity. One of these mechanisms may be associated with the endogenous hormone melatonin (9), which is produced by the pineal gland with a marked circadian rhythmicity (25). Circulating levels of melatonin are highest during the night and almost non-existent during the day demonstrating a tight correlation to the day/night cycle. Melatonin is thought to act as a chemical signal for darkness thereby helping to keep an animal entrained to the environmental light/dark cycle. *In vivo* experiments have demonstrated that administration of exogenous melatonin can phase-shift circadian rhythms upon acute administration, or lock circadian rhythms upon chronic administration in the absence of other environmental cues about day and night.

Although the link between melatonin production and circadian rhythmicity has been recognized for years, the sleep promoting effects of melatonin have yet to be unambiguously established and a significant difference of opinion exists among experts in the field (26-28). A review of the available clinical studies concluded that there is some evidence that melatonin may have modest efficacy in insomnia (29). However, it was noted that the studies completed to date have limited numbers of subjects and rely heavily on subjective patient reports rather than objective data. Despite the equivocal results to date, there remains a general perception that melatonin has a positive effect on sleep quality which has led some experts in the field to publish general guidelines for its use in the treatment of sleep disorders (30).

It has been proposed that melatonin may have a positive effect on sleep either by correcting circadian phase abnormalities due to its phase-shifting effects or by a direct, albeit modest, soporific effect (27). The effects of melatonin on circadian rhythmicity and therefore presumably on sleep are thought to be mediated by high affinity receptors found in the suprachiasmatic nucleus (SCN) (31). This is particularly significant because the SCN is generally regarded to be the site of the "biological clock". Two subtypes of receptors with high affinity for melatonin, mt_1 and MT_2, have been identified in SCN (32,33). Prior to the standardization of the nomenclature, these receptors were also referred to as mel_{1a} and mel_{1b}, respectively (34). The mel_{1c} receptor has not been found in mammals and remains outside of this nomenclature system.





9 X = OMe Y = H R = Me
10 X = OMe Y = H R = nPr
11 X = OBz Y = H R = M
12 X = H Y = CH_2Ph R = Me
13 X = H Y = CH_2Ph R = nBu

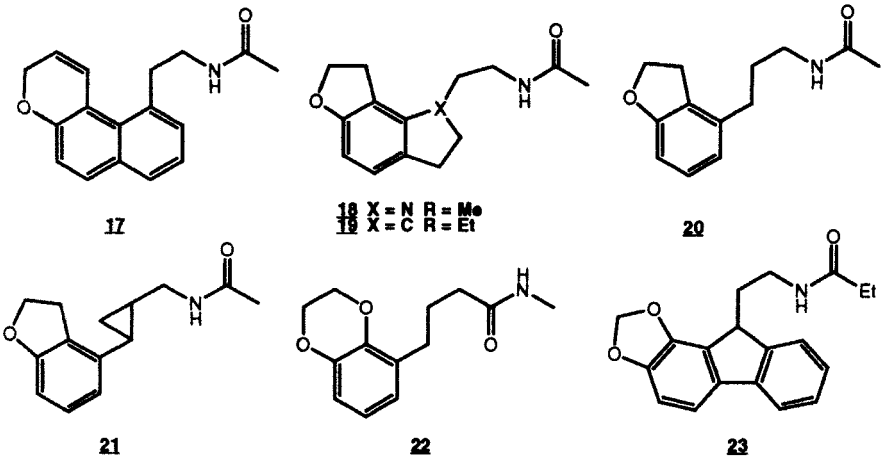
14 R = Me X = H
15 R = Et X = $OCOCH_2Br$

16

The development of high affinity ligands for melatonin receptors has progressed significantly during this decade. Prior to 1990, the only ligands known for melatonin receptors were simple derivatives of melatonin like 6-chloromelatonin. Since then, numerous laboratories have reported novel ligands for melatonin receptors belonging to a wide variety of structural classes. Although different researchers used different tissue preparations for binding studies and the exact nature of the melatonin receptor subtype(s) present in these tissues was not always known, the combined data have helped to elucidate a general, composite SAR for melatonin receptors (35). In brief,

both the methoxy group and the amide function are critical components for melatonin's binding affinity. Small increases in the size of the amide group (*e.g.* butyramides **10**) can be accommodated and substituents at the 2-position are well tolerated. The indole ring appears to be a scaffold for positioning the methoxy and amide groups but it does *not*, in and of itself, appear to be critical for melatonin receptor recognition. The indole ring has been successfully replaced by a variety of ring systems, most notably the naphthalene ring system. The naphthalene derivative S-20098 (**14**), which is currently in clinical trials, possesses essentially equivalent affinity to melatonin for the receptor in ovine *pars tuberalis*. In fact, the minimal structural features necessary for receptor recognition are contained in the phenylalkyl amide **16** which demonstrates remarkably good affinity for the receptor in chicken brain despite its simplicity. This structural motif is embedded in all melatonin receptor agonists reported to date. This area has been reviewed a number of times recently so an extensive review of all of the structural modifications known will not be repeated here (35-38). In addition, several modeling studies have been reported which describe pharmacophore models for melatonin receptors based upon an analysis of the structure/affinity data for a large number of the reported ligand chemotypes (39-42).

Studies have also determined that there is a definite conformational preference for the position of the oxygen lone pairs on the methoxy group as evidenced by the high affinity of the cyclic derivative **17** (K_i = 0.1 nM for receptor in chicken brain) (43). Another recently reported example is GR196429 (**18**) which is a high affinity agonist for human mt_1 and MT_2 receptors with pK_i values of 9.9 and 9.8, respectively (44,45). This has become a major theme in the recent patent literature. Patents for the cyclic ether derivatives of a number of previously reported melatonin ligands (**19**-**21**) have been published (46-48). Additional heteroatoms (e.g. **22** and **23**) can also be tolerated in the ring system (49,50). The 1,3-dioxolfluorene **23** is reported to have good affinity (IC_{50} < 25 nM) for the human mt_1 receptor.



17 **18** X = N R = Me **19** X = C R = Et **20**

21 **22** **23**

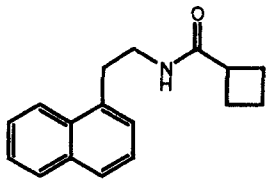
While the field has made good progress toward understanding and developing high affinity ligands for melatonin receptors, few ligands exist that are selective for melatonin receptor subtypes and this is clearly the challenge for the future. The low availability of selective ligands has hampered efforts to determine which receptor subtype (mt_1 or MT_2) is responsible for mediating the chronobiotic properties of melatonin. However, progress toward this end is being made. Studies in knockout mice, which lack a functional mt_1 receptor, have demonstrated that these animals still show a chronobiotic response to melatonin (33). Melatonin-induced phase shifts in the firing rate of the SCN are only modestly altered in these mt_1-receptor-deficient animals

but pertussis toxin will still block the phase shift. This implies that a G-protein coupled receptor other than the mt_1 receptor mediates the chronobiotic response. Although selective ligands are not abundant, selective antagonists for the MT_2 receptor have been identified (51). It has recently been demonstrated *in vivo* that these selective MT_2 -antagonists can block the phase advance in activity rhythms produced by acute administration of melatonin in mice (52). This complements the mt_1-knockout work by demonstrating that blocking the MT_2 receptor alone can block a chronobiotic response.

These results will have major implications for the field if they can be verified. To aid in this effort, additional agonists and antagonists with high selectivity for the various melatonin receptor subtypes are necessary. Some progress is beginning to be made in this area, primarily related to selectivity for the MT_2 receptor subtype. The first systematic comparison of the structure-affinity relationship for melatonin derivatives at the cloned mt_1, MT_2, and mel_{1c} receptors indicates that the SAR trends are similar for all of the receptor subtypes (53). However, while the methoxy group is the preferred substituent at the 5-position for both the mt_1 and MT_2 receptors, the reduction in affinity due to the removal or replacement of this group was far more dramatic for the mt_1 receptor than the MT_2. Due to this phenomenon, it was found that 5-benzyloxy N-acetyltryptamine (**11**) is 18-fold selective for the mt_1 over the MT_2 and can be considered as the first selective melatonin receptor agonist.

Another study has compared the effect of the length of the N-acyl side-chain on affinity for the human mt_1 and MT_2 and *Xenopus laevis* mel_{1c} receptor subtypes in a series of agonists and a series of antagonists (54). As has been well documented in the SAR literature, increasing the N-acyl sidechain from one to three carbon atoms was well-tolerated in the agonist series, but further lengthening dramatically reduced affinity. In contrast, affinity did not change substantially for the antagonist series typified by the structure of luzindole **12**. This implies that the N-acyl groups of the agonists and antagonists do not fit into the same pocket which might lead to important differences in SAR. This work identified an MT_2-selective antagonist from the luzindole series (**13**) with improved affinity and selectivity relative to the parent compound (89-fold selective over mt_1). An interesting tool has been reported for studying the MT_2 receptor (55). The naphthalene derivative **15** is reported to be an affinity ligand that specifically alkylated MT_2 receptors but not mt_1 receptors expressed in CHO cells.

Most of the known ligands for melatonin receptors are agonists. There is a significant need for high affinity antagonists and recent reports indicate that more effort is being expended in this area. An extensive SAR study on naphthalene derivatives has been reported which identifies **24** and a number of related derivatives as antagonists (56). A novel series of 2-[N-acylaminoalkyl]indoles has been reported to contain partial agonists (**25**), competitive antagonists (**26**) and putative inverse agonists (**27**) at the mt_1 receptor (57). Affinity for the other receptor subtypes was not reported. A derivative from a previously reported series of phenalene melatonin ligands (**28**) has recently been characterized as a putative antagonist of melatonin receptors (58).



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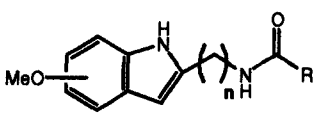
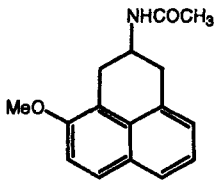


25 4-OMe n = 2 R = Et
26 4-OMe n = 1 R = Et
27 2-OMe n = 2 R = Me

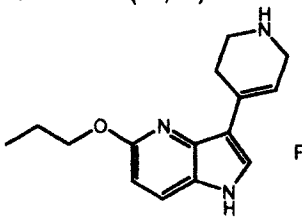


28

Serotonin Receptor Ligands – There is accumulating though sometimes conflicting data showing that serotonin (5-HT) affects circadian rhythmicity and sleep. Brain 5-HT

levels follow a circadian pattern, although it is still unclear whether this rhythm is endogenous or exogenous (59). The SCN receives serotonergic projections from the raphe nuclei that are involved in regulating circadian rhythms in hamsters (60). 5-HT potentiates light-induced phase shifts in rats and serotonergic agents elicit phase shifts in the timing of wheel running activity in rats and in the neuronal firing rate of the SCN. 5-HT receptors have been classified into seven families and at least 14 subtypes based on amino acid sequence homology, signal transduction mechanisms and functional characteristics (61,62). Receptor subtype selective ligands are being used to study the role of 5-HT in sleep and circadian regulation (63).

The 5-HT_1 and 5-HT_7 receptor families are closely related. 5-HT_1 receptors inhibit adenylate cyclase while 5-HT_7 receptors activate it. 5-$HT_{1/7}$ agonists mimic the effect of light on melatonin production in rats, indicating that 5-HT pathways may be involved in mediating the effects of light on the SCN (64). 8-OH-DPAT, an agonist selective for the 5-HT_{1A} receptor, produces a circadian response in the behavior of rats kept in constant darkness (65). 5-HT_{1A} agonists also increased nocturnal melatonin concentrations (66). The 5-HT_{1B} selective agonist, CP-94,253 (**29**) significantly increased waking and reduced slow wave sleep (SWS) and rapid eye movement (REM) sleep in rats. Pretreatment with the mixed $5HT_{1A/1B}$ antagonist pindolol reversed the effect on waking and SWS while REM sleep remained suppressed (67). 5-HT_{1B} selective agonists TFMPP (**30**) and CGS-12066A (**31**) inhibited light-induced phase shifts in a dose dependent manner. The inhibition was not affected by the 5-HT_{1A} antagonist WAY-100135 or by the 5-HT_2 antagonist mesulergine, but was attenuated by the 5-$HT_{1A/1B}$ antagonist methiothepin (68). Studies with 5-HT_7 selective agonists, such as SDZ-216525 (**32**), indicate that the 5-HT_7 receptor plays a major role in mediating the effects of 5-HT on photic responses in hamster SCN cells (69,70). The first 5-HT_7 antagonist, SB-258719 (**33**), was recently reported and others, such as DR4004 (**34**), have followed (71,72).



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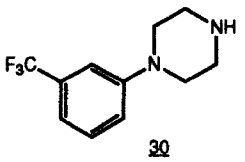

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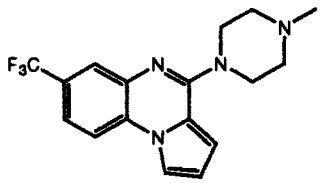

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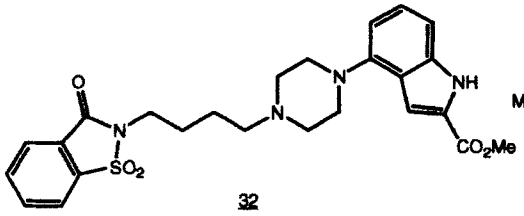

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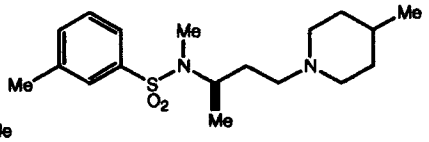

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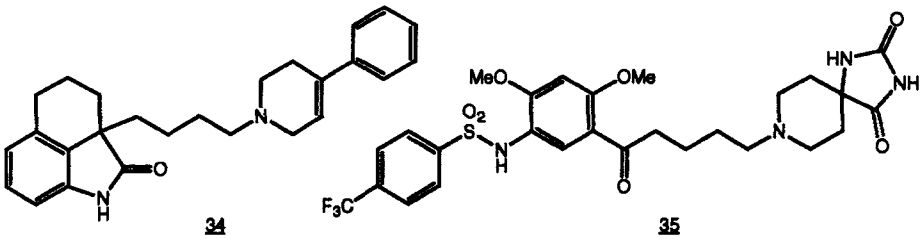
5-HT_2 receptors also inhibit adenylate cyclase. 5-HT_2 antagonists ritanserin and ketanserin were compared in the regulation of SWS in humans. Based on the relative affinities and the degree of effect the authors suggest that SWS is regulated primarily by 5-HT_{2C} receptors (73). 5-HT_{2C} agonists inhibited melatonin production acutely and phase-delayed production for several hours on subsequent nights. Ritanserin, a mixed antagonist, attenuated the effect. Ligands for 5-$HT_{1A/7}$ receptors failed to affect melatonin production. Agonists at the 5-HT_{2C} receptor have also been shown to mimic the effect of light pulses on some circadian rhythms in rats (75). Recently, the first 5-HT_{2C} selective antagonist, RS-102221 (**35**), was described (76).



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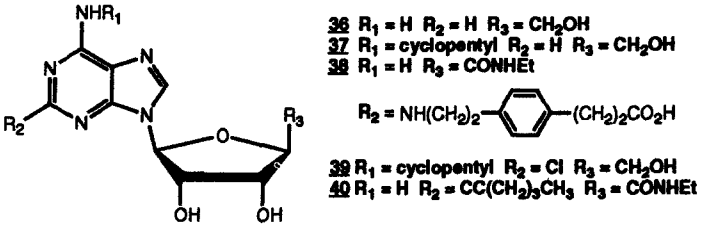
The 5-HT_3 receptor is coupled to a cation-selective ion channel. The 5-HT_3 agonist *m*-chlorophenylbiguanide increased wakefulness and REM latency while reducing SWS, REM sleep and the number of REM periods. Pretreatment with the 5-HT_3 antagonist MDL 72222 prevented these effects (77).

Adenosine Receptor Ligands - Common experience tells us that prolonged wakefulness decreases vigilance and is accompanied by an increasing drive toward sleep. This observation is supported by experimental evidence showing that both the propensity to sleep and the intensity of delta EEG waves upon falling asleep are proportional to the duration of prior wakefulness (78,79). It is thought that this effect of prior wakefulness may be mediated by an endogenous "sleep factor" that accumulates during wakefulness and is discharged during sleep. The inhibitory neuromodulator adenosine (36) is of considerable interest as a potential sleep factor because of pharmacological studies demonstrating sedative effects for adenosine agonists and powerful activating effects for caffeine and theophylline, known adenosine antagonists. However, *in vivo* data to support this proposed role for endogenous adenosine have only recently become available. *In vivo* microdialysis studies of extracellular levels of adenosine in the neostriatum and hippocampus of freely moving rats showed that maximal levels of adenosine were achieved during the rat's active period (80). In another microdialysis study using freely behaving cats, extracellular concentrations of adenosine exhibited progressive increases in the basal forebrain cholinergic region during sustained, prolonged wakefulness and declined slowly during sleep (81).



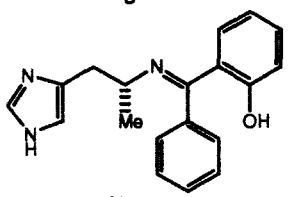
36 R_1 = H R_2 = H R_3 = CH_2OH
37 R_1 = cyclopentyl R_2 = H R_3 = CH_2OH
38 R_1 = H R_3 = CONHEt
R_2 = $NH(CH_2)_2$-(p-phenyl)-$(CH_2)_2CO_2H$
39 R_1 = cyclopentyl R_2 = Cl R_3 = CH_2OH
40 R_1 = H R_2 = $CC(CH_2)_3CH_3$ R_3 = CONHEt

Four adenosine receptor subtypes are known, A_1, A_{2A}, A_{2B}, and A_3. The current state of knowledge for these receptor subtypes, including the known SAR and the opportunities for drug development have been recently reviewed (82). Data exists to support a role for both A_1 and A_{2A} receptors in sleep. For example, i.p. administration of the selective A_1-agonist N^6-cyclopentyladenosine (CPA) 37 suppressed REM sleep in rats and enhanced EEG slow-wave activity in non-REM sleep. The effect on slow-wave activity was described as remarkably similar to the response to 6-h sleep deprivation. Direct administration of the highly selective A_{2A} agonist CGS21680 (38) directly into the subarachnoid space underlying the rostral basal forebrain of rats produced significant increases in the total amounts of non-REM sleep and REM sleep (83). The A_1 selective agonist CPA did not produce a significant increase in either type of sleep. It should be noted, however, that despite mounting evidence for a role of adenosine in sleep modulation, the specifics are still ambiguous and conflicting data exists which must be sorted out. For example, one group has reported that neither the selective agonist CCPA (39) nor the mixed agonist 2HE-NECA (40) significantly

affected sleep patterns in rats after ip administration (84). A discussion of the methodological issues that may contribute to the variability in results is given.

Histamine Receptor Ligands – Sedation is a well-known, though usually undesired, side-effect of antihistamines. This effect, coupled with the existence of histamine (HA) containing cells in the hypothalamus suggest a role for HA in the regulation of the sleep-wake cycle. *Lesions of the posterior hypothalamus produce sleep in several* mammalian species. Inhibition of histidine decarboxylase has been shown to impair the waking of rats. HA levels display a circadian rhythm and high levels are associated with high locomotor activity. Recent work has focused on elucidating the role of each of the histamine receptor subtypes on sleep. The area has been reviewed (85,86).

2-(3-Trifluoromethylphenyl)histamine, an H_1-selective agonist, increased wakefulness and reduced SWS in rat. Pretreatment with pyrilamine prevented the effects (87). The H_2 antagonists cimetidine and ranitidine did not affect wake time, total sleep or REM sleep in healthy humans (88). The H_3 agonist (*R*)-α-methylhistamine increased SWS and decreased wakefulness and REM sleep. Pretreatment with thioperamide, an H_3 antagonist, prevented the effects (89). BP2.94 (**41**), an H_3 agonist, also increased SWS. The effect was blocked by pretreatment with the antagonist carboperamide (90). Sch50971 (**42**), an orally active H_3 agonist, decreased locomotor activity and increased total sleep (91). Histamine itself has been shown to produce a phase delay in the circadian rhythm of neuronal firing in hamster hypothalamus slices containing the SCN. H_1 and H_2 antagonists had no effect on firing indicating involvement of H_3 receptors, but NMDA antagonists blocked the phase delay (92).



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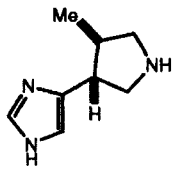

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Neuropeptides. Several neuropeptides appear to participate in the regulation of sleep and/or circadian behavior (93). In particular, there is a significant amount of recent literature devoted to the circadian effects of neuropeptide Y (NPY). NPY has been shown to phase shift circadian rhythms. NPY has also been shown to block $GABA_B$-induced (94), 5-HT-induced (95), and light-induced (96) phase shifts. Both the Y_2 and Y_5 have been reported to play a role in regulation of rhythmicity (97,98). In one study, both NPY and 5-HT were necessary for treadmill running entrainment of mice (99).

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Chapter 6. Progress in the Development of Neurokinin Antagonists

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Introduction - The neurokinins substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are related neuropeptides that are widely distributed in the peripheral and central nervous systems (CNS). With the recent development of highly selective non-peptide antagonists of these receptors, it has become possible to investigate the physiological roles of these peptides. The vast majority of these compounds are selective SP antagonists however a number of selective NKA and NKB antagonists have been reported. In recent years, neurokinin (NK) receptor antagonists have been the subject of intense interest by pharmaceutical companies because of their potential utility for the treatment of a wide variety of medical disorders. Many of these compounds have been reported to show good activity in pre-clinical assays relevant to pathological conditions, but until recently clinical data to support these uses was limited. However, in the last year, important new data on the clinical properties of SP antagonists (SPAs) have become available.

Following a catalogue of the range of therapeutic targets described in the literature, in particular relating to CNS disorders, this chapter highlights the recent progress in the development of NK antagonists concentrating on work reported since the last review in Annual Reports in Medicinal Chemistry (1).

THERAPEUTIC TARGETS

Pre-clinical findings suggest the use of NK antagonists in a diverse range of therapeutic indications, including, migraine (2), arthritis (3), pain (4) and inflammatory diseases such as cystitis (5). Other indications include, inflammatory bowel disease and other gastric disorders (6), emesis, in particular cancer chemotherapy induced emesis (7), asthma (8), anxiety and depression (9), cancer (10), schizophrenia (11), glaucoma and ocular hypotension (12), neuronal injury and stroke (13), cardiac disorders (14), and psoriasis (15).

The possible role of NKs in CNS disorders has been reviewed recently (16). In fibromyalgia patients serum levels of SP and serotonin have been correlated with pain intensity and sleep disturbance (17). In addition a number of studies have implicated NKs in spinal cord pain transmission (18). In mice lacking the preferred NK$_1$ receptor for SP there is an alteration in nociception, stress-induced analgesia and aggression, suggesting that SP modulates responses to pain and other stressors (19). In the rat striatum, SP facilitates dopamine release and metabolism (20), suggesting a modulatory interaction with monoamines. A novel role of SPAs in affective disorders has been highlighted in a recent landmark publication of clinical and preclinical findings (21).

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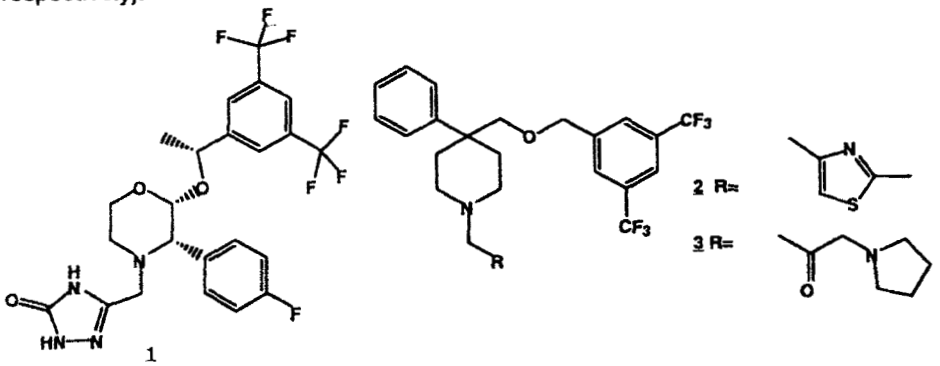
NK_1 RECEPTOR ANTAGONISTS

Chemistry - The design and synthesis of the morpholine acetal L-754,030 (MK-869) 1 has been described (22) together with details of the SAR in this series. The SAR for a series of 4,4-disubstituted piperidine benzylethers 2, 3 has been reported (23); replacement of the ether oxygen by carbon reduces affinity. Compounds 2 and 3 had good oral kinetics in rats and were active in an assay of resiniferatoxin-induced vascular leakage in guinea-pigs (ID_{50} values were 0.28 and 0.22 mg/kg p.o. respectively).



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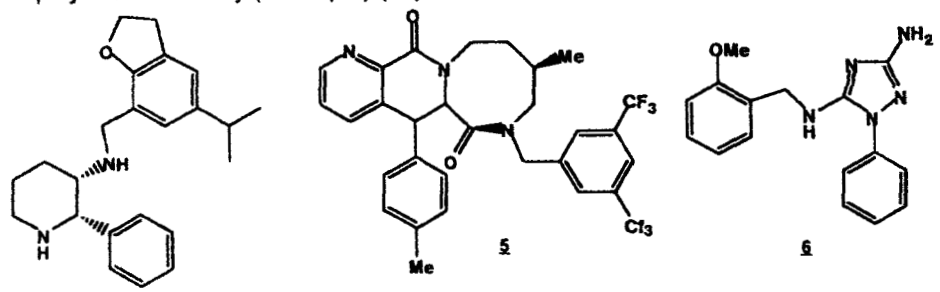
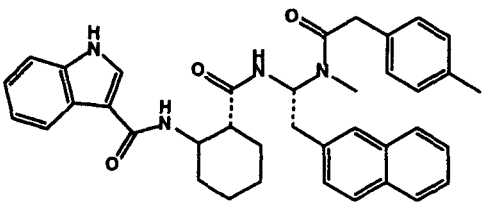
The benzyl amine HSP-117 (4) has been reported as a potent SPA with good anti-emetic activity (24). In an elegant stereochemical study (25) it was shown that the SPA 8-oxo-1,7-naphthyridine-6-carboxamide (5) exists as separable and stable (*R*)- and (*S*)-atropisomers originating from the restricted rotation around the -C(6)-C(:O)- bond. Among the four isomers the (a*R*,*S*)-enantiomer (5) exhibited the most potent antagonistic activities with an IC_{50} value of 0.80 nM and ED_{50} values of 9.3 μg/kg i.v. and 67.7 μg/kg p.o. for inhibition of capsaicin-induced plasma extravasation in guinea pig trachea. A novel class of heterocyclic achiral SPAs have been reported 6 that display modest affinity (Ki 2.4 μM) (26).



4 5 6

MEN 11149 is a pseudopeptide (7) that is a highly selective SPA (pKi 8.5), with a very slow disassociation from the receptor, that dose dependently inhibits (Sar^9, $Met(O_2)^{11}$0) SP-induced plasma protein extravasation in guinea-pig bronchi (ID_{50} = 0.22mol/kg i.v.; ID_{50}=0.97 mol/kg p.o.) (27).



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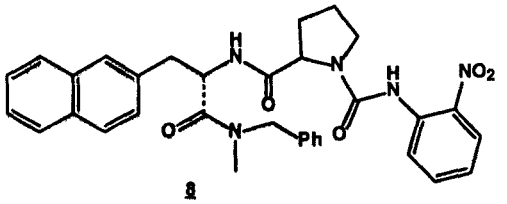
SDZ NKT 343 (8), is the result of an optimization effort starting from a sub-micromolar screening lead 9. Key steps in the optimization (28) were replacement of methoxy by halogen, followed by modification of the proline diphenylmethyl amide to afford the 2-chlorobenzylthiourea (10). Subsequent introduction conversion to a phenylurea led to 8 (NK1 Ki = 0.16 nM).



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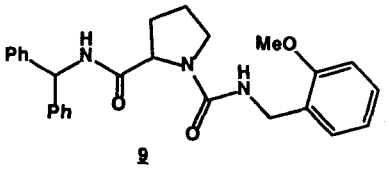

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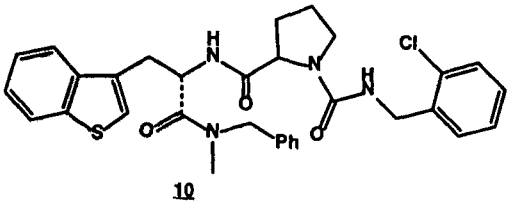
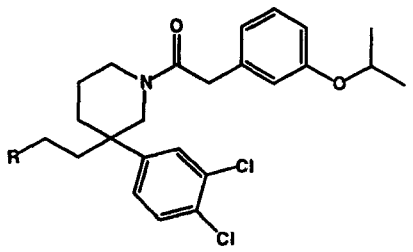
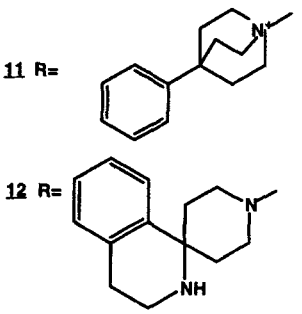


10

Starting from SR-140333 (11) a series of spiro-substituted piperidin-1-yl(ethyl)piperidines (12) were prepared and shown to be potent SPAs with potent inhibitory activity against selective NK1 receptor agonist-induced bronchoconstriction in guinea pigs (29).





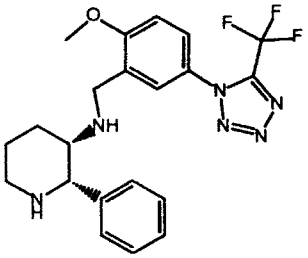
11 R=

12 R=

Preclinical biology – Evaluation of the preclinical pharmacology of SPAs has been complicated by species variants in NK_1 receptor pharmacology and most compounds are unsuitable for *in vivo* studies using rats, the most commonly used preclinical species. In ferrets, SPAs exhibit broad spectrum anti-emetic activity (30,31) suggesting that they may have wider clinical efficacy than existing anti-emetic treatments. Further studies indicate that an important site of action is the nucleus

tactus solitarius, an emetic centre in the brainstem (24,30). Interestingly, SPAs also ameliorated both radiation and cisplatin-induced tissue damage (32). A number of studies have explored the role of SP in the control of pain; comparison of brain penetrant and non-penetrant compounds is consistent with a central site of action in the spinal dorsal horn for the anti-nociceptive effects of SPAs (33,34).

In addition to its localisation in sensory afferents and in brainstem emetic nuclei, substance P has a widespread distribution throughout the brain and there has been speculation about the possible use of antagonists to treat psychiatric disorders. The rationale that SPAs might be useful as antipsychotic drugs has been built on evidence that SP modulates the activity of the mesolimbic dopamine system through which established antipsychotic drugs are thought to act (35). There is growing evidence that SP is involved in affective disorders. SP and its preferred NK_1 receptor are widely expressed throughout the fear-processing pathways of the brain, including the amygdala, septum, hippocampus, hypothalamus and periaqueductal gray (36). Activation of the SP pathways by direct central injection of agonists produces a range of fear-related behaviours and defensive cardiovascular changes in animals (37,38).



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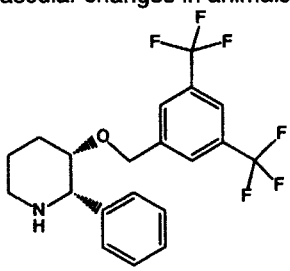
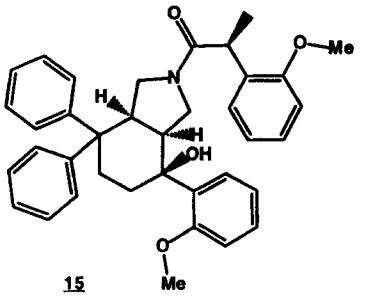


14

There is preliminary evidence that SPAs may antagonise certain stress responses. GR205171 (13), a compound with nanomolar affinity for the rat NK_1 receptor, was able to inhibit the increase in DOPAC levels in the prefrontal cortex caused by 20 min of restraint stress in rats; this effect was shown to be enantioselective, and resembled that seen with the benzodiazepine anxiolytic, diazepam (39). Using c-Fos immunohistochemistry, it has been shown that i.c.v. infusion of the SPAs inhibited neuronal activation of the locus coeruleus caused by immobilisation stress in rats (40). In guinea-pigs, central infusion of a SP agonist elicited a vocalisation response that was inhibited enantioselectively by the SPA L-733,060 (14) (3 mg/kg s.c.) (21). Interestingly, the vocalisations were also inhibited by pretreatment of animals with the antidepressant drugs imipramine and fluoxetine, and this was the first evidence of a similarity in the profile of a SPA and antidepressant drugs. The studies were extended to demonstrate that SPAs, like clinically used anxiolytic and antidepressant drugs, caused enantioselective and complete inhibition of vocalisations elicited by transient maternal separation of guinea-pig pups from their mother (21), confirming an important role of endogenously released SP in this stress response.



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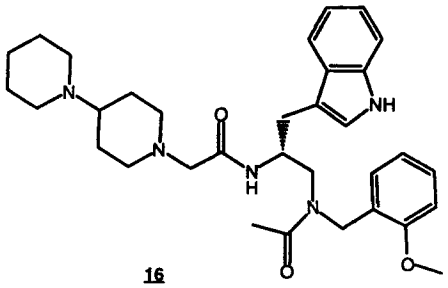
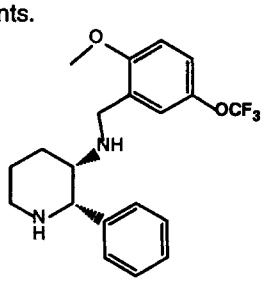


16

Clinical Studies - RPR 100,893 a perhydroisoindole (15) at doses of up to 20 mg (41) and LY 303,870 (lanepitant, 16) at doses of 30, 80 and 240 mg p.o. (42) have previously been reported to be ineffective for the treatment of acute migraine. However, these compounds have poor CNS penetration (43) that may have contributed to their lack of efficacy. Recently, results of further clinical trials with more potent, brain penetrant compounds have been disclosed. Compound 13 is a high affinity NK_1 receptor antagonist and recent PET studies in monkeys (44) showed that it rapidly penetrates the CNS. Migraine patients were treated with 10 (25 mg i.v.) or placebo for a single migraine attack. No pain relief was experienced by either group at any time point. L-758,298 a phosphate pro-drug of the high affinity, and brain penetrant NK_1 receptor antagonist 1. Up to 60mg (i.v. infusion) of the pro-drug or placebo was administered to migraineurs upon experiencing a moderate or severe headache; however, there was no evidence of pain relief (45).

The studies describing the evaluation of 17 for the treatment of post-operative nausea and vomiting were reviewed previously (1); further studies using the quinuclidine CJ-11,974 (18) have now been reported (46). Acute emesis after Cisplatin treatment was controlled in 86% of patients receiving 100 mg b.i.d. 18 for five days in combination with a $5\text{-}HT_3$ antagonist and dexamethasone. Emesis was also prevented in 68% of patients in the delayed phase, a symptom that is poorly controlled by currently available therapies. Clinical data (47) with compound 1 has also reported, of patients receiving five daily doses of 1 (400mg p.o.), delayed emesis was completed prevented in 83%. Remarkably, in patients who received only a **single** dose of 1 79% had no delayed emesis, whilst acute emesis was controlled in 93% of patients.



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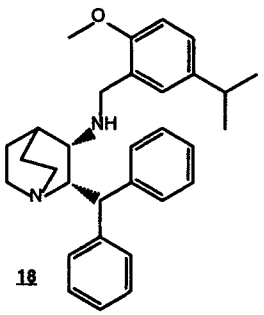


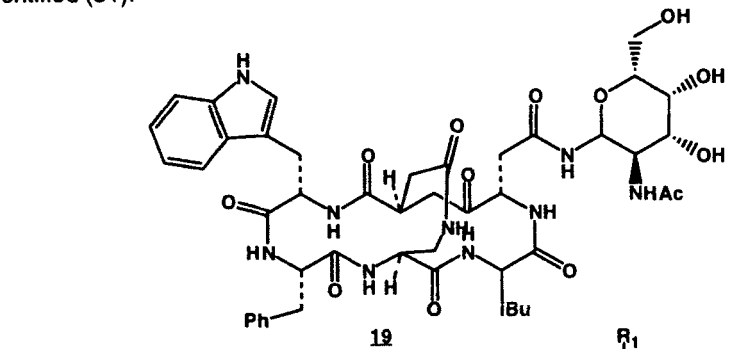
18

The most exciting discovery in this area was the report that a SPA was safe and effective in the treatment of outpatients with major depressive disorder and moderately high anxiety levels (21). Compound 1 was chosen to test the concept clinically because its high affinity, good brain penetration, long duration and oral bioavailability permitted once daily-oral dosing. Patients received 1 (300 mg/day), paroxetine

(20mg/day) or placebo and were assessed during 6 weeks of treatment using the 21-item Hamilton Depression Rating Scale. The results showed that 1 was as effective as paroxetine in reducing depression and anxiety in this patient population; importantly, the SPA was extremely well-tolerated and, unlike paroxetine, was associated with significantly fewer reports of sexual dysfunction. An exploratory, clinical trial with 1 (400 mg/day for 3 weeks) was also initiated in schizophrenic. Preliminary results of this study indicate that this compound lacks efficacy in ameliorating the core symptoms of acute psychosis (48).

NK_2 RECEPTOR ANTAGONISTS

Chemistry - Details of the metabolism and pharmacokinetics of MEN 11420 (nepadutant, **19**) in rat have now been published (49). The plasma half life after i.v. administration is 44 min, and it has <3% bioavailability p.o. The first non-peptide NK_2 antagonist SR 48968 (saredutant, **20**) has provided the starting point for a number of selective NK_2 antagonists (**21**) and a number of mixed antagonists (see below). An investigation of the SAR of the piperidine substitution (50) has demonstrated wide tolerance to different substitution, in particular simple tertiary carbinols have equivalent affinity and *in vivo* activity in the NKA-induced dyspnea screen (2-4 μmol/kg). YM-35375 highlights an alternative piperidine substitution (**22**) that has also been identified (51).



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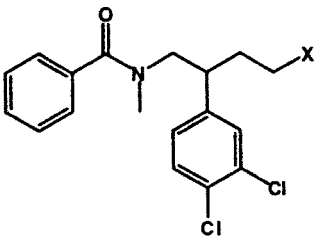
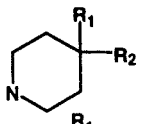
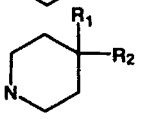


20 X=



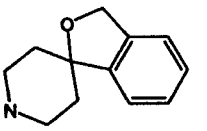
R_1=NH_2; R_2=Ph

21 X=

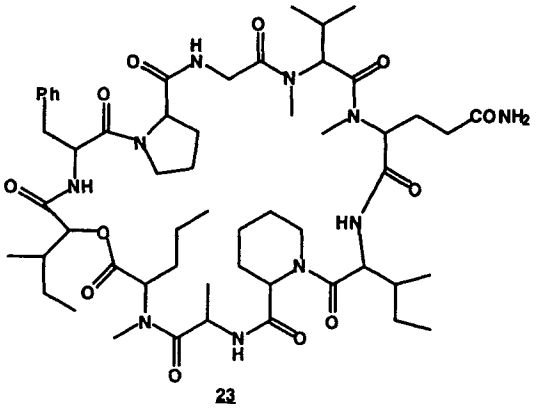


R_1=H; R_2=$COH(Et_2)$

22 X=



Whilst the majority of work has focused on non-peptide antagonists the cyclodepsipeptide SCH 217048 (**23**) has also been reported (52).



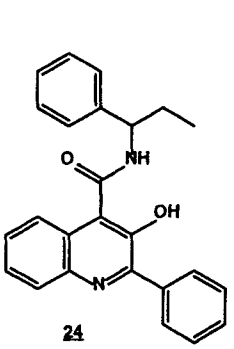
23

Preclinical biology – Expression of NK_2 receptors in the mammalian CNS has not yet been established, and so the focus here has been to explore the effects of antagonists in peripheral tissues such as the lungs. Both **19** and **20** produced a dose-dependent inhibition of bronchoconstriction induced by [Bala8]NKA-(4-10) or vagal stimulation (53). However much of the work in this area is now focussing on the use of dual NK_1/NK_2 antagonists (see below).

Clinical Studies -In a recent double-blind clinical trial examined the effect of **20** on NKA-induced bronchoconstriction, a dose of 100mg, p.o. or placebo was administered to 12 patients suffering from mild asthma and the NKA challenge was performed at 1.5 and 24h after dosing. The treated group reported a significant inhibition of bronchoconstriction (54). This is the first clinical evidence to support the utility of a selective NK_2 receptor antagonist for the treatment of asthma.

NK_3 RECEPTOR ANTAGONISTS

Chemistry - Full details of the structure activity relationships of a series of 2-phenyl-4-quinolinecarboxamide NK_3 receptor antagonists has been reported (55), optimization with respect to metabolic stability afforded SB223412 (**24**).



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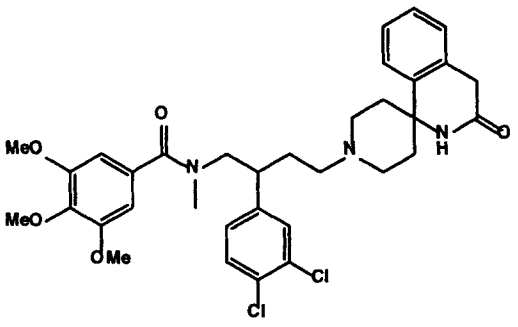


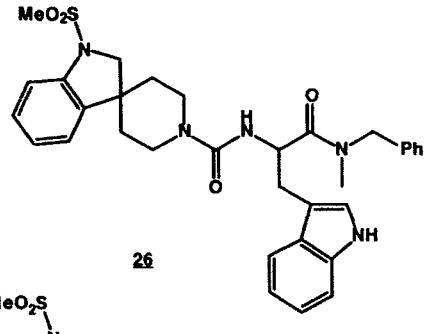
25

Preclinical biology – There has been much interest in the possible uses of NK_3 receptor antagonists for CNS disorders; however, interpretation of preclinical findings is complicated by marked phylogenetic differences in the expression of NK_3 receptors in the CNS, which appears to be considerably higher in rat brain than in human brain

(56). The role of NK_3 receptors in pain transmission has not been studied as extensively as the NK_1 receptor. Recently, NK_3 receptors have been located on capsaicin-sensitive primary afferent neurones in the spinal cord (57) and SR142801 (**25**) was found to potently inhibit depolarisations of the ventral root caused by application of NK-B or stimulation of the dorsal root of spinal cords from neonatal rats (58). Other preclinical evidence that NK_3 receptors modulate central dopamine function in rodents has raised speculation that NK_3 antagonists may be clinically useful for the treatment of schizophrenia; compound **25** is in clinical trials for this indication. Studies investigating the combination of an opioid antagognist with NK_3 agonists and antagonists suggest a possible neurokinin-opioid relationship in the modulation of experimental. anxiety in mice (59)

MIXED ANTAGONISTS

Chemistry - The NK_2 selective antagonist **20** also served as a starting point for a series of dual NK_1/NK_2 antagonists (60). Conformations in which the aryl rings of the N-methylbenzamide and 3,4-dichlorophenyl moieties are close to each other through a cis-amide bond, have high affinity for the NK_1 receptor. Also a hydrogen bond-accepting group in the spiro-substituted piperidine moiety may be crucial for exhibiting high affinity for the NK_2 receptor. YM-44778 (**26**) showed high and well-balanced affinity for NK_1 and NK_2 receptors. In contrast, starting from an NK_1 receptor antagonist it has also been possible to identify high affinity dual NK_1/NK_2 antagonists (61). The structural features **27** responsible for binding to both NK_2 and NK_3 receptors have also been explored (62).



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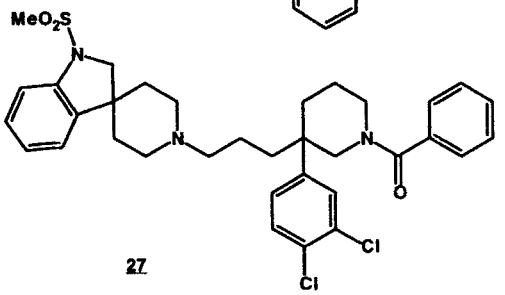

27

Preclinical biology - Whilst NK receptors are widely distributed it is in the treatment of asthma (63) that the use of mixed antagonists has been investigated. The pulmonary effects of tachykinins are produced by actions on NK_1 and NK_2 receptors and methods to evaluate the contribution of each of the tachykinins have been reported (64). The role of NK_3 receptors in gastric secretion have also been investigated (65).

Conclusion - Whilst preclinical studies have suggested a variety of different clinical utilities it is only recently that this potential has come to fruition. The successful clinical trials in the treatment of emesis, depression and early promise for the treatment of asthma underline the enormous potential for this class of compounds. With the identification of potent and selective antagonists further clinical utilities will undoubtedly be identified.

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SECTION II. CARDIOVASCULAR AND PULMONARY DISEASES

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Chapter 7. Recent Approaches Towards Inhibiting Allergic Eosinophilia

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Introduction - Airway hyperreactivity to a variety of bronchospastic mediators is a cardinal hallmark of asthma, and correlates with the severity of the disease (1). The airways of asthmatic patients are infiltrated by inflammatory leukocytes, of which the eosinophil is the most prominent component (2). The magnitude of acute and chronic asthmatic reactions correlates with the number of eosinophils present in the lung, and suppression of eosinophil accumulation attenuates development and progression of these processes (3). Although asthma is the most common disease associated with eosinophilia, there are less prevalent eosinophilic disorders including atopic dermatitis, idiopathic hypereosinophilic syndrome, pemphigus, conjunctivitis and certain neoplasms such as Hodgkin's disease (4).

Eosinophils are present in the blood of all vertebrates down the phylogenetic scale. They are produced from myeloid precursors in the bone marrow in response to a variety of cytokines, and are released from the bone marrow in response to an appropriate stimulus (5). Once in circulation, eosinophils rapidly accumulate in tissue in both the human and animal models. Although there are very few eosinophils in the lung of normal individuals, they accumulate dramatically in the lungs of people with both allergic and non-allergic asthma. Eosinophils appear to be more involved with the late phase reaction of asthma than the immediate bronchoconstrictive reaction to allergen (6).

Eosinophils are capable of lysing and activating cells and destroying tissues (7). When activated, they synthesize and release inflammatory cytokines such as IL-1, GM-CSF, IL-3 and TGF-α, and inflammatory mediators such as PAF, LTD_4, and reactive oxygen species that can produce edema, bronchoconstriction, and chemotaxis (7). Concurrently, they secrete a set of cytotoxic enzymes and proteins such as eosinophilic cationic protein and major basic protein that can lyse epithelial tissue and are found in the lungs of asthmatic patients (7). Eosinophils may provide protection against parasitic infection but, unlike neutrophils, they play no other significant defensive functions. Thus, it should be possible to inhibit the actions of eosinophils without the severe immunosuppressive consequences of inhibiting the actions of other inflammatory cells such as neutrophils, macrophages or lymphocytes. Consequently, the eosinophil appears to be an ideal target for inhibiting tissue damage that accompanies pulmonary inflammation during asthma.

Potential approaches to control allergic eosinophilia are numerous due to the large variety of mediators that have been implicated in their regulation. Some of these include PAF, LTB_4, fMLP, C5a, several interleukins and GM-CSF, but many of these approaches have either been unsuccessful or produced disappointing clinical findings (8). Recent efforts have focused on eosinophil activation and recruitment by somewhat more specific mechanisms, including their induction and maturation by IL-5 and subsequent migration and infiltration into the airways via VLA-4 and CCR3.

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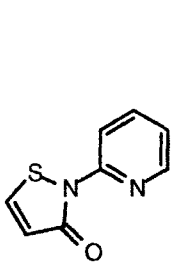
NOVEL AGENTS DISCOVERED TO REGULATE ALLERGIC EOSINOPHILIA

Regulation of IL-5 - Growth, maturation and survival of human eosinophils involve several cytokines including IL-5, IL-3 and GM-CSF. IL-3 and GM-CSF stimulate the production of other leukocytes such as neutrophils, while IL-5 is selective for the eosinophil/basophil lineages. Thus, by inhibiting IL-5, it should be possible to selectively suppress these two cell types that are associated with allergic disorders (9). Corticosteroids have many effects but inhibition of release, migration, chemotaxis and activation of eosinophils, probably through suppression of IL-5 synthesis at the level of gene expression, may be a critical element in their proven efficacy in asthma (10). Accordingly, efforts to selectively regulate IL-5 synthesis or its function have been pursued in recent years (11).

Blocking the action of IL-5 with a monoclonal antibody to IL-5 inhibits bronchoalveolar eosinophilia and airway hyperreactivity, caused by allergic challenge in mice, guinea pigs and monkeys (12-14). Therefore, anti-IL-5 antibodies should have therapeutic utility for the treatment of asthma and several are undergoing clinical development (15). Sch-55700 is a humanized monoclonal anti-IL-5 antibody being developed for the treatment of asthma and is currently in Phase I clinical trials (16,17). A human monoclonal antibody, SB-240563, is being developed by another group and is in phase II clinical trials for asthma (18,19).

IL-5 exerts its biological actions through a high affinity receptor which is composed of two subunits, designated α and β (20). While the α component is responsible for IL-5 binding specificity, the β subunit is essential for signal transduction, and is shared by the receptors for GM-CSF and IL-3 (20). Therefore, the IL-5 receptor α subunit is a alternative drug target and antibodies to the α subunit have been claimed in a recent patent application (21).

Although viewed as a challenge in drug discovery, blocking protein-protein interactions with small molecular weight antagonists is another possible approach for inhibiting the binding of IL-5 with its receptor. Screening libraries of compounds has produced little success for this approach, although there have been some reports on compounds that inhibit the binding of IL-5 to the α subunit of its receptor. A series of isothiazolones irreversibly inhibit IL-5 binding, as exemplified by isothiazolone **1** which exhibits an IC_{50} of 20 μM in the binding assay (22). The irreversible inhibition is proposed to result from the formation of a disulfide adduct between cys-66 of the α subunit with the isothiazolone. Two structurally unrelated compounds, **2** and **3**, were more recently reported to inhibit IL-5 receptor binding (23). As with **1**, these compounds bind irreversibly to the receptor with similar affinity (IC_{50}'s = 11 and 8 μM, respectively). Although not reported for isothiazolone **1**, compounds **2** and **3** also inhibit GM-CSF and IL-3 dependent cell proliferation. The reactivity and poor selectivity of these agents preclude them as significant leads for further investigation.



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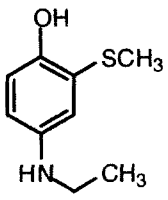

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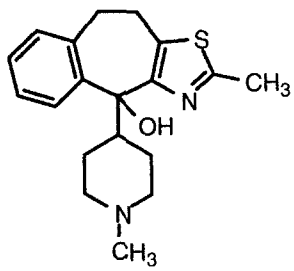
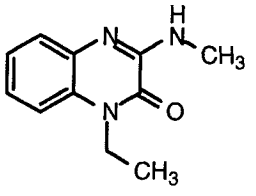


3

A series of linear and cyclic peptides have been disclosed which bind to the IL-5 receptor (24-28). Specifically disclosed peptides are high in molecular weight and contain at least ten or more amino acid residues. Peptides containing replacements for the amide linkages are also claimed, presumably to circumvent metabolic issues, but no data is presented.

The crystal structure of IL-5 has been reported and the important binding residues have been identified using site-directed mutagenesis (29-31). As additional information on the exact nature of the binding of IL-5 to its receptor is gathered, designed libraries utilizing this structural information may offer the greatest possibility for success via this approach.

Owing to the difficulties and challenges of inhibiting IL-5 function by blocking activation of its receptor, regulation of IL-5 biosynthesis is an alternative approach that has been pursued. Selective regulation of IL-5 biosynthesis or activation may provide therapeutic agents with diminished side effects over corticosteroids. Although many compounds have been reported to inhibit IL-5 levels *in vivo*, they often have additional effects on other cytokines and their mechanism of actions are largely unknown. Examples of such compounds include F-11105 (**4**) which is in phase I clinical trials for asthma (32). It inhibits IL-5 secretion along with that of TNF-α, IL-4 and GM-CSF from human cells. An interesting series of azauracil derivatives were recently reported to inhibit IL-5 production in human blood (33). Compound **5** inhibited IL-5 synthesis *in vitro* by 93% at 1 μM.



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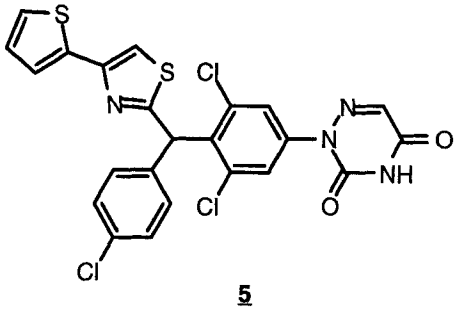
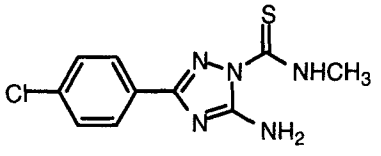


5

A series of triazoles have been reported to inhibit eosinophilia in a model of Sephadex G-200-induced hyperresponsiveness in which IL-5 was demonstrated to play a role (34). GCC-AP0341 (**6**) inhibits IL-5 stimulated human eosinophil survival with an IC_{50} of 2 μM and blocked eosinophilia in the sephadex model with an ED_{50} of 0.3 mg/kg po. As a follow-up to these derivatives, a series of triazolotriazine thione analogs were prepared and **7** was found to be similar in efficacy to **6** (35). Interestingly, compound **7** was weaker than **6** in its inhibition of antigen induced hyperresponsiveness in guinea pigs, possibly suggesting that eosinophil recruitment is not the only critical factor in determining airway hyperreactivity (36).



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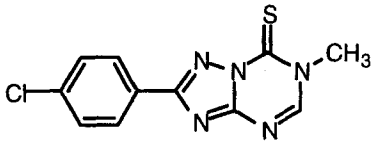


7

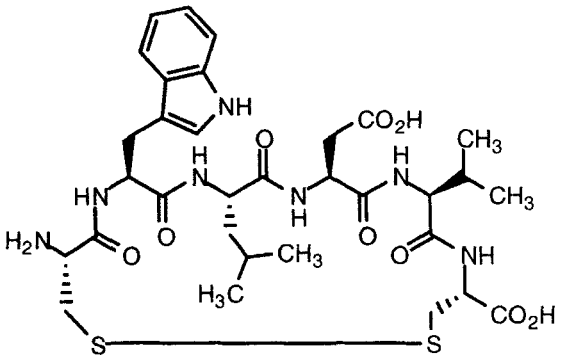
Many additional compounds have been reported which affect eosinophilia, but the mechanisms by which their inhibition occurs is largely unknown.

Inhibitors of VLA-4 ($\alpha 4\beta 1$) - While IL-5 is involved in the release of eosinophils from the bone marrow, the subsequent migration and localization of these inflammatory cells into airway tissues is controlled by other mediators. A variety of adhesion molecules or integrins specific to the various leukocytes are activated and expressed at different stages of the adhesion cascade, and thereby, enable these cells to adhere to the surface of tissues. Selective accumulation of eosinophils into specific tissues such as lungs requires, among others, initial interactions of eosinophils with the endothelium and subsequent migration into the inflammatory site. These interactions are mediated through specific integrin molecules on eosinophils and their counterligands on the endothelium. The integrin, known as VLA-4, is a non-covalent heterodimer consisting of two subunits, $\alpha 4$ and $\beta 1$, and is localized on eosinophils, monocytes and lymphocytes, but not on neutrophils (37). VLA-4 mediates eosinophil adhesion and migration by binding to either of its counterligands, VCAM-1 or fibronectin. VCAM-1, a member of the immunoglobulin super family, is rapidly induced on endothelial cells in response to IL-1 and TNF-α and is greatly increased at sites of inflammation (38). The VLA-4 interactions with VCAM-1 mediate the initial capture and rolling of eosinophils comparable to selectins on neutrophils. Accordingly, specific blockage of this interaction should enable inhibition of eosinophilia without affecting neutrophils, and thereby, may be useful in the treatment of allergic diseases without the degree of immunosuppression associated with neutrophil inhibition (39,40).

As with IL-5 monoclonals, antibodies to VLA-4 have been generated to block the binding of VLA-4 to its natural ligands (41). Reflecting the specificity for eosinophil recruitment, these antibodies to VLA-4 inhibit lung eosinophilia, but do not affect neutrophils (42). Antegren™ is a humanized monoclonal antibody which specifically binds to the $\alpha 4$ subunit of VLA-4 (43). It is in phase II clinical trials for the treatment of multiple sclerosis and inflammatory bowel disease (44).

The limitations associated with the therapeutic utility of monoclonals has encouraged the discovery of smaller molecule antagonists of VLA-4. Many of the lower molecular weight antagonists initially disclosed are peptides based on the amino acid sequence of fibronectin, or more specifically, the CS-1 sequence of fibronectin (45,46). Specifically, a three amino acid sequence, LDV (i.e., Leu-Asp-Val), was postulated to be the minimal recognition site for VLA-4 binding to its counterligands (47). Based on this observation, a variety of cyclic and non-cyclic peptides were designed to mimic this sequence.

The cyclic hexapeptide, TBC 722 (**8**), was proposed to conformationally mimic the LDV sequence on fibronectin and inhibited VLA-4's binding to VCAM-1 with an IC_{50} of 0.75 μM (48). However, a recent study by McIntyre and coworkers suggests that a mechanism independent of direct competitive binding may be responsible for the inhibition of integrin signaling (49). Another series of cyclic derivatives based on the LDV sequence were reported by another group (50). In general, compounds with fewer than six amino acid residues were the most potent with **9** appearing to be the best compound reported. A series of cyclic peptides in which the termini are joined by a variety of different linking moieties have also been disclosed (51). These



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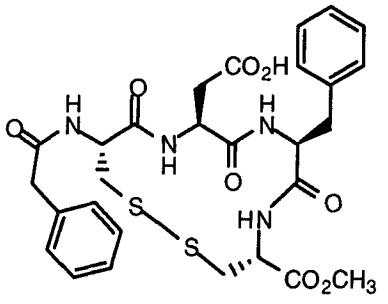
derivatives are also based on the LDV sequence, as exemplified by **10**, but no biological data is presented. Dimeric compounds which display the same sequences in duplicate have also been reported (52).



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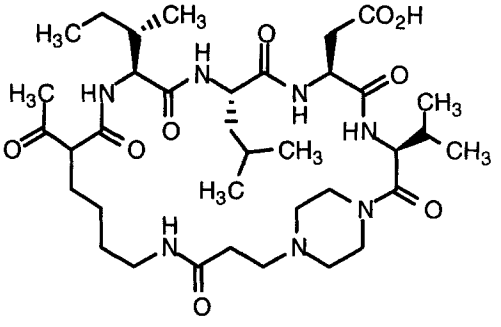
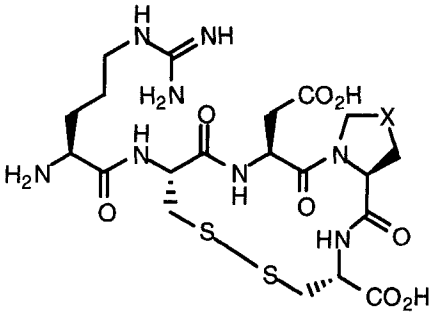
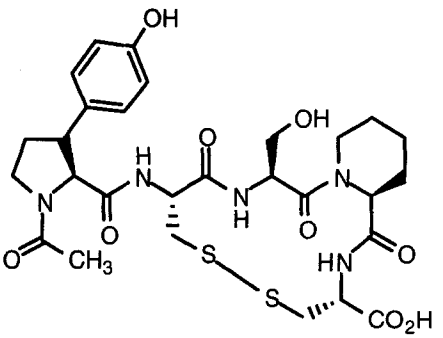


10

Another amino acid sequence present on fibronectin is also known to be a common binding motif found in many integrin ligands. This three amino acid sequence, RGD (i.e., Arg-Gly-Asp), has also been exploited in the design of inhibitors of VLA-4. The cyclic pentapeptide **11** was shown to mimic this binding motif, but with low affinity (53). Since the VLA-4/VCAM-1 binding interaction is relatively weak like most integrin interactions, an SAR study around **11** was explored to improve the affinity of the antagonists (54). Accordingly, modifications of the N-terminus and the DP (i.e., Asp-Pro) sequence of **12** provided a series of cyclic peptides that exhibit higher affinity for VLA-4 than its normal ligand VCAM-1. The hydroxy phenyl derivative **13** was found to be 500-fold more potent than the normal VLA-4/VCAM-1 interaction.

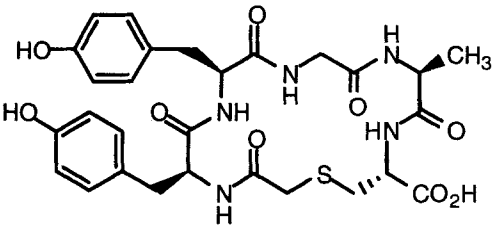


11 X = S
12 X = CH_2



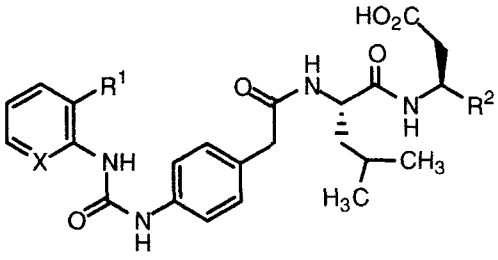
13

Another series of cyclic thioethers, linear peptides and their dimers have been reported that are largely derived from the essential amino acid sequences of Antegren™ and several other related anti-VLA-4 antibodies. Several of these compounds, as exemplified by **14**, are reported to inhibit VLA-4/VCAM-1 binding with IC_{50}'s ≤ 50 μM (55).

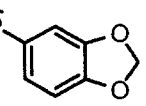


14

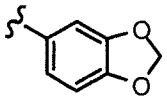
In an effort to reduce the molecular weight and complexity of these initial VLA-4 antagonists, a series of non-cyclic small peptides were reported by several groups. These compounds are also based on the LDV sequence and contain a variety of substituents on their N-terminus. The N-substituents are reported to impart a degree of specificity for molecular binding to VLA-4 over other integrins (56-58). Based on these results a hypothetical model of the inhibitor/VLA-4 interaction was devised and used to discover a series of potent compounds, exemplified by Bio-1050 (**15**) (IC_{50} = 30 nM), which contain a bis-aryl urea as the N-substituent. From this series Bio-1211 (**16**) (IC_{50} = 3 nM) and Bio-1218 (**17**) (IC_{50} = 1 nM) have been mentioned as possible development candidates, one of which is being developed in an aerosol formulation (59-61).



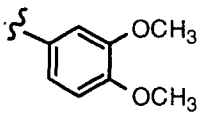
15 X = CH, R^1 = H, R^2 =



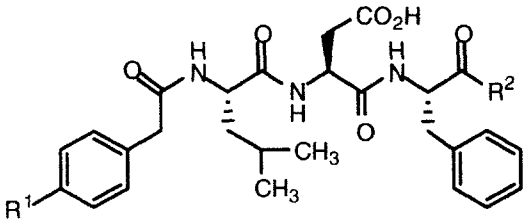
16 X = CH, R^1 = CH_3, R^2 =



17 X = N, R^1 = H, R^2 =



Structurally similar compounds, which also contain a bis-aryl urea moiety, have been reported by another group. These compounds inhibit VLA-4 binding in the sub-micromolar range (e.g., **18**, IC_{50} = 0.3 μM) and differ from **15** - **17** predominately at their C-terminus (62). More recently, this group disclosed a series of compounds that further explore both the N- and C-termini of the LDV sequence suggesting that the substituents can be significantly altered (e.g., **19**) (63). A structurally related compound, **20**, has been shown to block late phase airway responses and airway hyperreactivity in Abraham's allergic sheep model (64).



19 R^1 = H,

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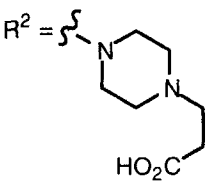
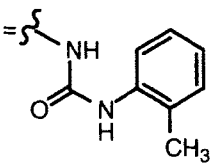


18 R^1 =



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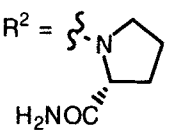
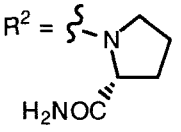


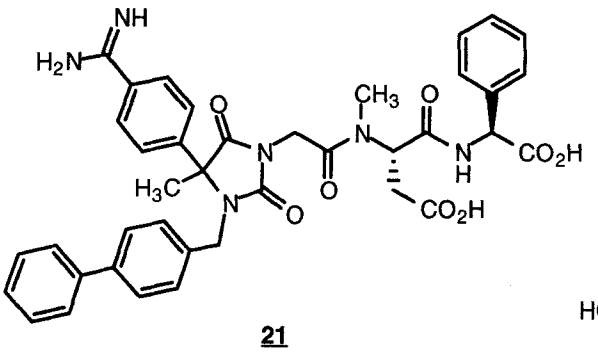
20 R^1 = H,

R^2 =



A series of patent applications disclosing compounds in which the N-terminus is substituted with a variety of structurally different 2,5-dioxoimidazolines has been reported. This modification has resulted in a fairly potent series of compounds, as exemplified by **21** (IC_{50} = 90 nM) (65-67).

In an effort to identify VLA-4 antagonists that are less-peptidic in nature, Ellman and coworkers constructed a library of β-turn mimetics that mimic the LDV sequence (68). Interestingly, compounds containing the closest mimic of this sequence by displaying hydrophobic moieties between an aspartic acid mimic were not active. The epimeric diacids **22** were identified as the most potent inhibitors with low micromolar binding, indicating the feasibility of discovering less-peptidic, lower molecular weight inhibitors of VLA-4.



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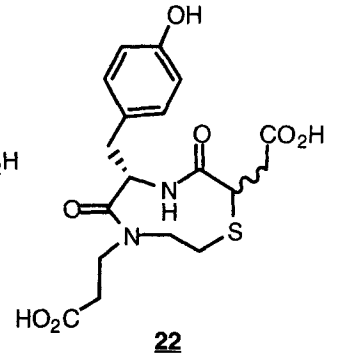
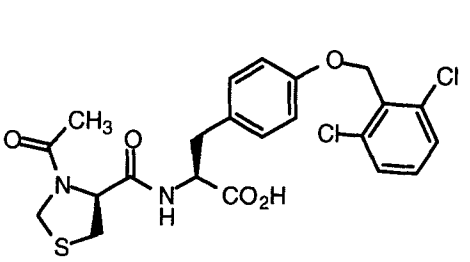


22

The possibility of discovering lower molecular weight less-peptidyl inhibitors of VLA-4 binding has been further realized in a number of recently published patent applications. A variety of structurally diverse antagonists are disclosed, as exemplified by **23** and **24**. Unfortunately, their potency relative to the peptidyl inhibitors is unknown, since no biological activities are reported (69-71).



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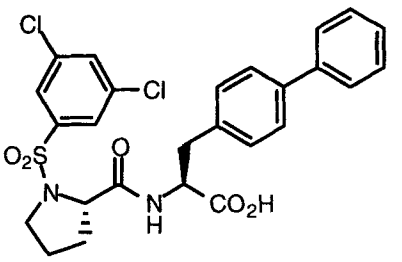


24

CCR3 Antagonists - The trafficking and activation of leukocytes throughout the body is largely regulated by chemokines and their receptors (72). Once adherent to adhesion molecules, eosinophils are acted upon by chemotactic agents such as chemokines produced by endothelial cells at sites of inflammation, thereby effecting their transendothelial migration. Chemokines represent a class of recently identified chemotactic cytokines and are divided into two distinct supergene families, CXC and CC, designated by the position of the first two cysteine amino acid residues. The CXC family of chemokines is primarily chemotactic for neutrophils, while the CC family is primarily chemotactic for eosinophils and mononuclear cells. The CC chemokines that are strongly chemotactic for eosinophils and are expressed at high levels in inflammed tissues include eotaxin, eotaxin-2, RANTES and MCP-3 (72,73). These chemokines act by specific interactions with CCR3, the predominant chemokine receptor on the eosinophil, and appear to play a major role in trafficking eosinophils into the airway tissues (74). It is noteworthy that eotaxin and eotaxin-2 act exclusively through CCR3, while RANTES and MCP-3 bind other chemokine receptors as well (72,73). Eosinophils also express CCR1, but the attractiveness of antagonizing CCR3 lies with the high predominance of this receptor on eosinophils

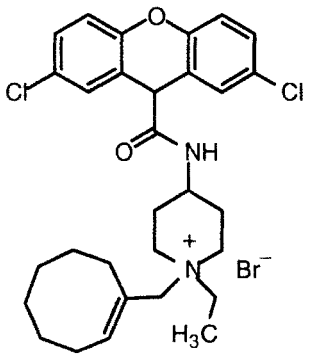
alone (75). By virtue of its being a critical component in the selective migration of eosinophils in tissue, CCR3, a seven transmembrane spanning G protein coupled receptor, may be an excellent target for drug discovery. The feasibility of discovering small molecule antagonists of such receptors is often promising, and recently, a limited number of patent applications have appeared disclosing novel compounds.

An interesting series of tricyclic derivatives that bind to CCR3 with high affinity were disclosed in a patent application (76). Overall, quarternary ammonium derivatives of the piperidine ring are the most potent with compound **25** having the highest receptor affinity [IC_{50} (CCR3) = 0.7 nM]. The corresponding neutral derivatives are weaker. The disclosed compounds appear to be antagonists, but they also bind to CCR1 receptors with high affinity [IC_{50} (CCR1) = 1.8 nM for **25**], thereby limiting their chemoattractant specificity.

Another patent application disclosed a series of piperidines and piperazines which bind to the CCR3 receptor with **26** (IC_{50} = 240 nM) exhibiting the highest receptor affinity of those reported (77). These compounds inhibit eotaxin mediated chemotaxis *in vitro* indicating that they are antagonists, but no data is given on their selectivity versus other chemokine receptors.

A series of compounds which block RANTES and MIP-1α binding to HL-60 cells, presumably via the CCR1 receptor, has been claimed in a patent application (78). Although binding to CCR3 was not reported, several compounds weakly inhibit eotaxin-induced eosinophil chemotaxis, indicating possible antagonism of the CCR3 receptor. L-377 (**27**) completely inhibited eosinophil chemotaxis at 3 μM and was significantly weaker in its inhibition of HL-60 chemotaxis (66% inh. at 10 μM).

A series of compounds structurally related to known neurokinin antagonists are claimed to bind predominately to CCR3 and/or CCR5 receptors (79). However, no biological activity is disclosed to access the binding affinities of these compounds for either of these receptors.



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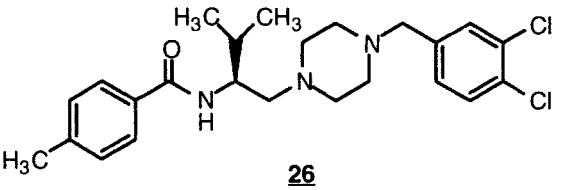

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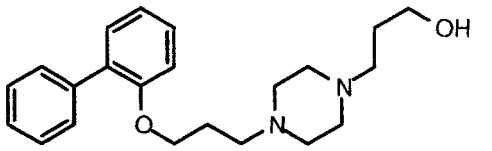

27

Conclusion - In the last few years new and more selective approaches for controlling allergic eosinophilia have emerged and progress has been made in discovering small molecule inhibitors for these new targets. As more potent and selective compounds with improved pharmacokinetics are discovered, clinical results with the various antibodies to these targets will help to define the role and potential benefits of these various approaches for treating allergic disease.

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Chapter 8. Pharmacological Therapy for the Treatment of Erectile Dysfunction

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Introduction - Erectile dysfunction (ED) is defined as the inability to achieve or maintain an erection sufficient for satisfactory sexual performance (1). The term erectile dysfunction has replaced the less precise impotence which has historically been used to describe a wide range of male sexual dysfunctions, including retarded ejaculation, low desire as well as the inability to achieve or maintain an erection. According to the 1992 NIH Consensus Statement an estimated 10-20 million U.S. males suffer from ED, with an incidence of 5% at the age of 40 rising to 15-25% at the age of 65 and above. More recently the Massachusetts Male Ageing Study found a combined prevalence of 52% in men aged 40-70 (2). In this case ED was very strongly associated with age as were certain medical conditions, such as diabetes, heart disease and hypertension.

Physiology of the Penis and Mechanism of Erection - The erectile tissue of the human male penis consists of the *corpus cavernosum* and the *corpus spongiosum*. The *corpus cavernosum* consists of two cylinders of sponge-like tissue, consisting of interconnected spaces (sinusoids) bounded by trabeculae consisting of smooth muscle lined with endothelium. The cavernosal bodies are bounded by the relatively indistensible *tunica albuginea*, which, by allowing pressure in the cavernosal bodies to build up to close to systolic pressure is a key factor in the erectile process. The *corpus spongiosum* contains very little blood when the penis is flaccid, but becomes dilated when engorged with blood. However, as the spongiosum is not bounded by an indistensible tunica, it does not contribute to rigidity. In the flaccid penis *cavernosal and venous blood pressures are almost the same, and the smooth muscles of the* penile arteries are contracted to maximise the resistance to arterial inflow. This tone is maintained by excitatory adrenergic innervation. During erection the pivotal, and rate-limiting, event is relaxation of the *corpus cavernosum* smooth muscle and the consequential inflow of blood to fill *cavernosal* spaces which increases pressure within these spaces so that the penis becomes erect (3). During an erection arterial blood flow to the penis increases 10 to 20-fold and the blood flows into the penis through a network of very small arteries that supply the erectile bodies, and this is a reason why atherosclerosis can also lead to ED. As important as the inflow of blood is to the production of an erection, the control of outflow by venous occlusion is critical to the control and maintenance of erection. Therefore problems such as venous leakage can also give rise to ED.

Erection is triggered and maintained *via* central nervous control through two different nerve pathways. Reflex erections are under spinal control and psychogenic erections under direct central control. In healthy males these two mechanisms are believed to work synergistically in the control of erections. Therefore any reduction of neuronal function by peripheral neuropathy associated with diabetes, for example, can also lead to ED. In addition, spinal cord injury can also contribute to the presence of ED through loss of central control, although the reflexogenic pathway still operates.

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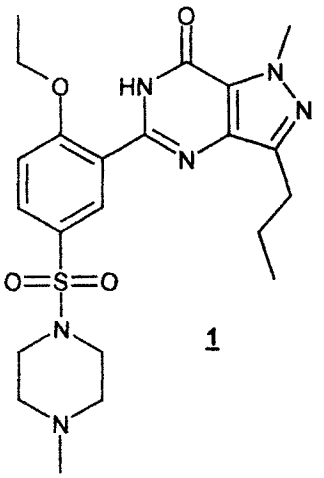
The causes of ED can be broadly classified into three classes; psychogenic, organic and mixed. By far the most common causes of organic impotence are vascular in origin (50-60% of all cases). These disorders include both cardiovascular and cerebrovascular diseases such as atherosclerosis, hypertension, stroke and peripheral vascular disease. The other main organic causes of ED are neurogenic or neurological. The most common diseases causing damage to the nerves supplying the penis are multiple sclerosis, spinal cord injury and peripheral neuropathy as a result of diabetes. In has been estimated that 50% of all male diabetics suffer from ED and that this patient group accounts for 30-50% of the total number of ED patients (4). Patients with primary psychogenic ED have all the normal penile physiology to allow them to have an erection but anxiety or other psychological factors can inhibit penile erection. The inhibition of an erection is under sympathetic control, with anxiety leading to an increase in tone, release of noradrenaline which leads to contraction of cavernosal smooth muscle and opposing the erectile process.

Drugs Approved for the treatment of ED - The pharmacological treatment of ED has recently been revolutionised by the approval, last year, of sildenafil (1) as the first oral therapy for ED (5). Sildenafil is a potent and selective cGMP-specific type 5 phosphodiesterase inhibitor (PDE5) and has rapidly become the first line therapy for the treatment of ED. Several reviews on its clinical efficacy have been published already and will not be summarised here (6). The mechanism of action of sildenafil in improving penile erection in men with ED is due to its inhibition of PDE5 activity in the *corpus cavernosum* (Fig. 1). Sexually stimulated penile erection is as a result of nitric oxide (NO) mediated *trabecular* smooth muscle relaxation (7-9). During sexual stimulation nitric oxide is released from nerves supplying the *corpus cavernosum* smooth muscle. The NO diffuses into penile smooth muscle cells and binds to the heme of soluble guanylate cyclase. This results in stimulation of cGMP synthesis which reduces intracellular calcium levels following binding to cGMP-dependent protein kinases and cGMP-dependent ion channels (10). This reduction in intracellular calcium results in *trabecular* smooth muscle relaxation leading to increased flow of blood facilitating the expansion of the *corpora cavernosa* leading to a functional erection (11 and 12). It has been shown that PDE5 is the major PDE isoform in human penile tissue from whole tissue extracts, however it has only recently been confirmed that the PDE5 activity was attributable to trabecular smooth muscle rather than from platelets in the tissue sample (13). Sildenafil also amplifies the neuronal NO/cGMP pathway involved in relaxation of human *corpus cavernosum* and therefore enhances the normal process leading to penile erection, suggesting that erections will not occur in the absence of sexual stimulation (14).



1

Why a Type 5 PDEI Enhances Erections

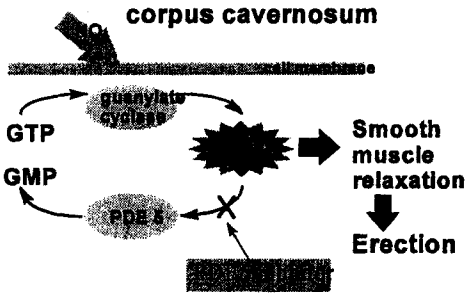
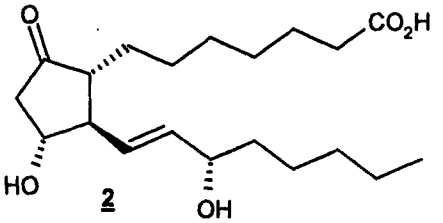


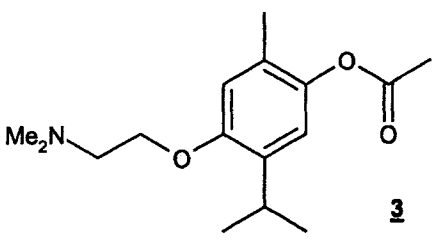
Figure 1

Prior to the introduction of sildenafil during 1998, the mainstay of ED drug therapy had been intracavernosal (*i.c.*) administration of vasodilators such as alprostadil (2, prostaglandin E_1), which is the only approved *i.c.* therapy. Formulations of 2 linked to α-cyclodextrin significantly improve aqueous solubility and stability and avoid the need for refrigeration.



2

Sexual stimulation is not required to initiate an erection using 2, as direct administration of prostaglandins to the *corpus cavernosum* induces a pharmacological erection. The erection continues after ejaculation and is maintained until the drug action wears off. Alternative, less invasive methods of delivering alprostadil to the *corpus cavernosum* have also been developed and a novel system to directly administer a pellet containing 2 into the penile urethra from which it is absorbed has found some utility. Other approaches involves *ic* administration of non-selective α_1-adrenoceptor antagonists such as moxisylate (3) and phentolamine (4) (or a mixture of (4) and papavarine) which decrease sympathetic tone and produce an erection after sexual stimulation (15). In contrast to the effects observed with 2, detumescence occurs after ejaculation.



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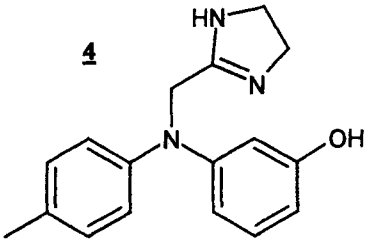

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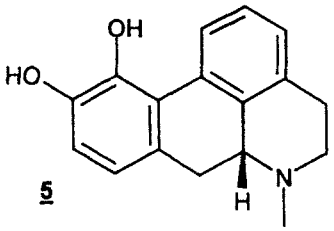
Oral/sublingual 4 is currently approved for use in the treatment of ED in Mexico and is currently undergoing wider regulatory review.

Central Control of Penile Erection - The dopamine receptor agonist Apomorphine (5) induces penile erections in normal patients after *s.c.* administration (0.25, 0.5, or 0.75

mg), and the anti-cholinergic, benztropine has no effect on apomorphine-induced penile tumescence (16). These data suggest that dopamine receptors play a role in normal erectile function and that dopamine-mediated penile tumescence is not modulated by cholinergic systems. Consequently dopamine receptors agonists may be useful in the treatment of ED .



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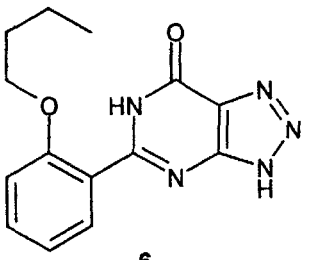
In light of these data sublingual apomorphine is being evaluated as a potential therapy for the treatment of ED (17).

INVESTIGATIONAL DRUGS FOR ED.

Novel PDE5 inhibitors - There continues to be great interest in the identification of novel PDE5 inhibitors with potential utility for the treatment of a range of diseases and in particular the treatment of ED. This chapter will focus on recently published PDE5 inhibitors, and information regarding historical research can be found in a number of earlier reviews (18-22). Considerable structural diversity has been found to be compatible with good PDE5 inhibitory activity and compounds based upon a wide range of structural starting points have been reported. These compounds have mainly been published in the patent literature suggesting that many are only in the early stages of clinical development. Compounds based upon zaprinast (6) and more recently the pyrazolopyrimidinone template of sildenafil (1) have formed the basis of new discoveries in this area. Replacement of the 5'-sulfonamide substituent found in sildenafil with an acyl moiety leads to 7 which potently inhibits human *corpus cavernosum* PDE5 (IC_{50} 6.4nM) (23). In addition, the morpholine derivative (8) also potently inhibits *corpus cavernosum* PDE5 with an IC_{50} of 1.9nM (24).



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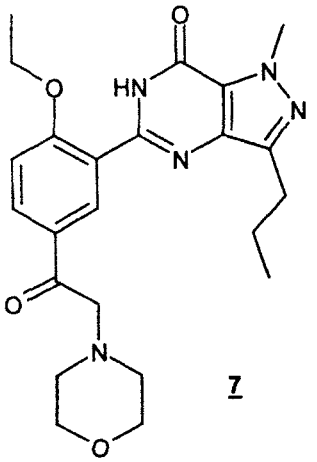

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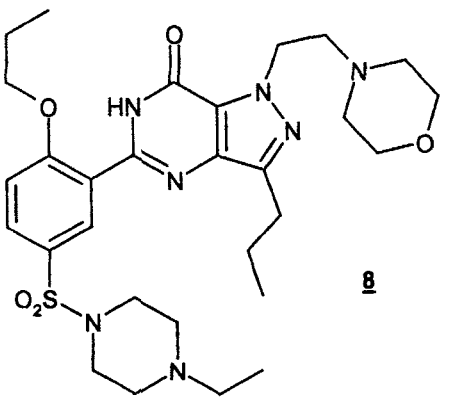

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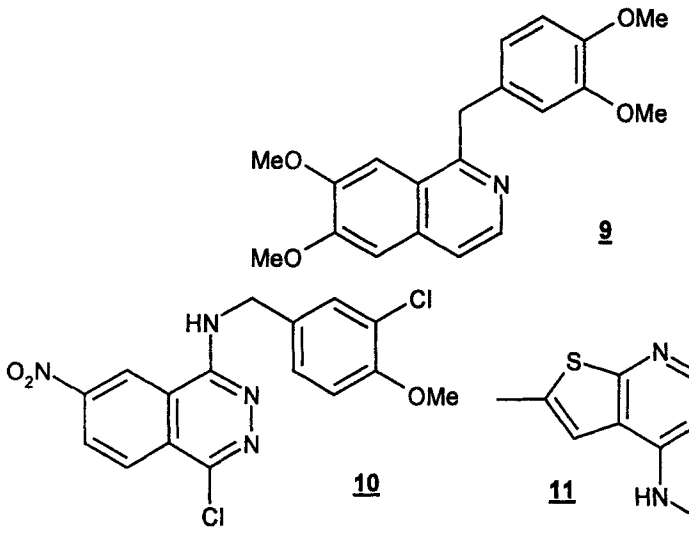
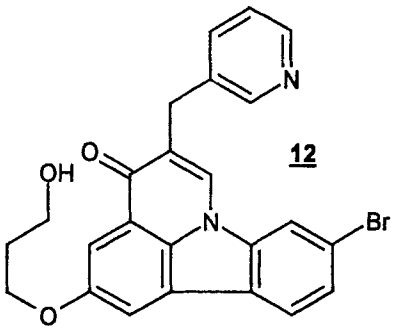
Papavarine (9) analogues are another area of continued interest, and (10) is a potent and selective inhibitor of PDE5 (IC_{50} 3.5nM) derived from porcine platelets (25). In general, the 6-substituted phthalazines were significantly more potent than the corresponding 7-substituted analogues. The 4-methoxy-3-chlorobenzylamino group is the preferred 4-substituent and polar electron withdrawing groups are favoured at the 6-position. Another class of compounds exemplified by (11) which appear to have been derived from papavarine have also been disclosed (26).

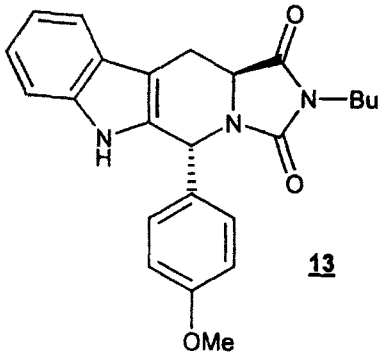


A recent patent application has disclosed a novel class of pyridocarbazolone PDE5 inhibitors. In particular, (12), is a potent inhibitor of PDE5 (IC_{50} 5.7nM) with selectivity over PDE1 and PDE3 (27).



A series of novel tetrahydro-β-carboline derivatives have been disclosed which exhibit good inhibitory activity against recombinant human PDE5 (28). Significant structural latitude is possible in this series with the closely related compounds (13) (IC_{50} 4nM), (14) (IC_{50} 7nM), and (15) (IC_{50} <10nM) all exhibiting excellent potency. Potency in this series seems to be unaffected by ring stereochemistry and N-alkyl substitution, and the aromatic group in the β-position of the carboline ring seems to require a *para*-

oxygen functionality for potency. Enlargement of the cyclic urea moiety to a diketopiperazine (16) also results in good levels of PDE5 potency (29-31).



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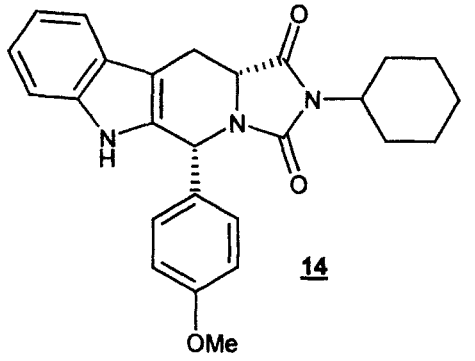

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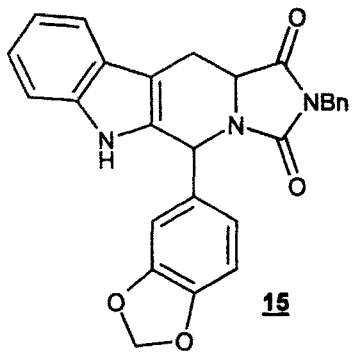

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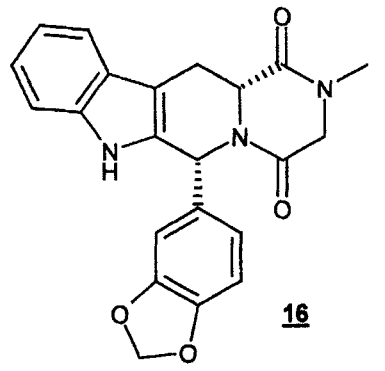
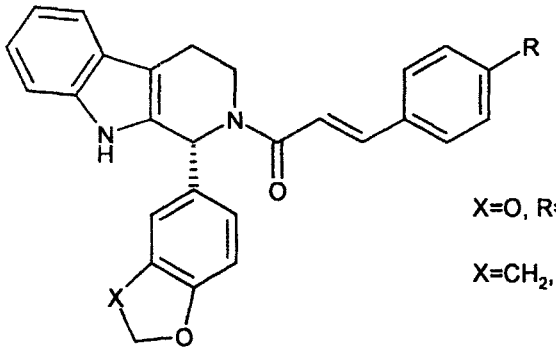


16

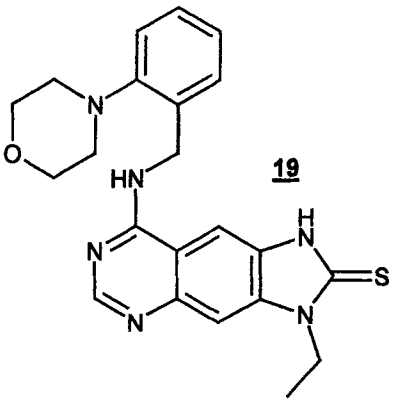
A closely related series of tricyclic β-carboline derivatives (17) (IC_{50} 2nM) and (18) (IC_{50} 2nM) have also been disclosed (32). Polar electron-donating and electron-withdrawing groups are equally well tolerated in the 4-position of the cinnamamide residue. In addition, the bicyclic oxygen containing heterocycles, benzdioxolane or a benzdihydrofuran, are preferred in the carboline β-position.



X=O, R= CO_2H (17)

X=CH_2, R= (18)

The imidazoquinazoline (19) shows 98% inhibition at a concentration of 1nM of dog trachea-derived PDE5 (33). Indole derivative (20) is claimed to be a PDE5 inhibitor but no specific activity is described (34).



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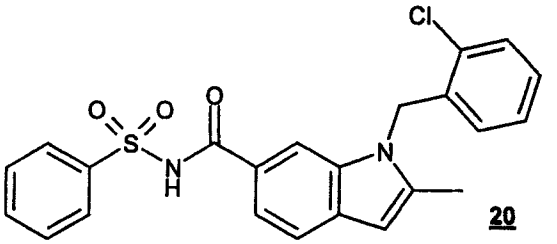

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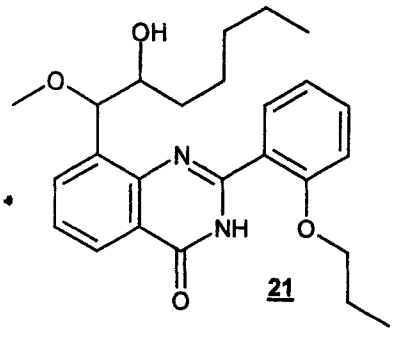
Non-selective PDE5 Inhibitors - A number of non-selective inhibitors of PDE5 have been reported. Quinazolinone (21) (IC_{50} 1μM), and purinones (22) (IC_{50} 1μM) and (23) (IC_{50} 0.1μM) are also active against PDE1 and PDE3 (35-37).

Polycyclic pyrazolopyrimidine (24) is a balanced PDE1/5 inhibitor (IC_{50} 60nM and 75nM respectively) with 700-fold selectivity over PDE3 (38).



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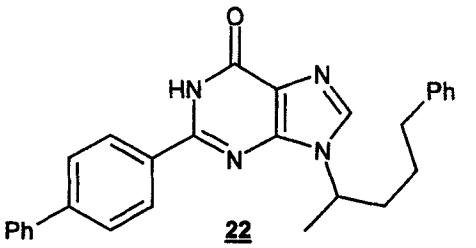

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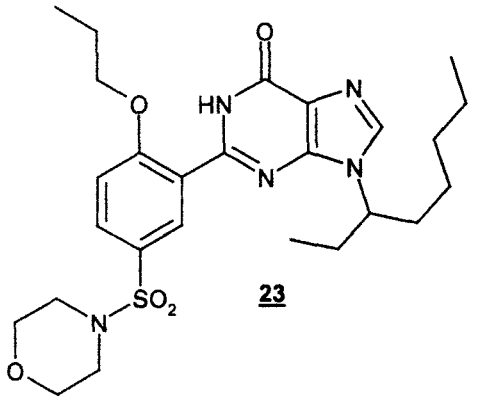

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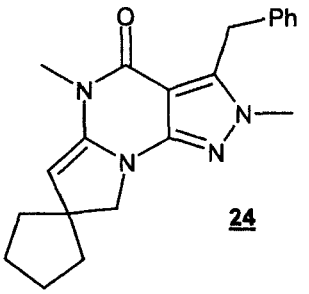

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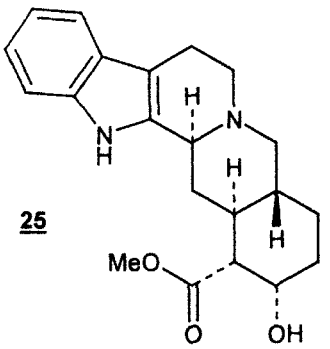
Other Non-PDE Based Investigational Agents for ED.

Yohimbine, **25**, an adrenergic receptor antagonist has also been investigated for ED, however there is conflicting data regarding its clinical efficacy. A meta-analysis of randomized clinical trials suggests that **25** has significant effects above placebo (39), whereas a 30-day trial with high-dose **25** (100mg, *p.o.*) in men with organic erectile dysfunction failed to show a significant difference compared to placebo (40). In addition, a randomized cross-over study of **25** after *i.c.* administration in men with vasogenic ED, failed to demonstrate efficacy (41). Delequamine (RS-15385-197, **26**) related structurally to yohimbine is a potent and selective α_2-adrenoceptor antagonist undergoing clinical evaluation for ED (42-44). Fluparoxan (GR-50360A, **27**) is a non-imidazoline, highly-selective antagonist of alpha-2 adrenergic receptors, both *in vitro* and *in vivo*, which is no longer being clinically investigated for depression, but is now being pursued for male sexual dysfunction (45). Atipamezole (MPV-1248, **28**) an imidazoline adrenergic antagonist has also been claimed to have potential for the treatment of erectile dysfunction, and was found to increase sexual behaviour in stumptail monkeys (46).



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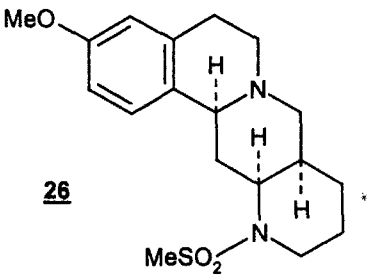

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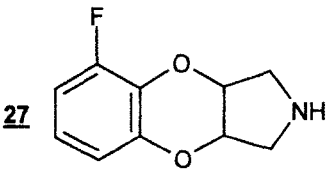

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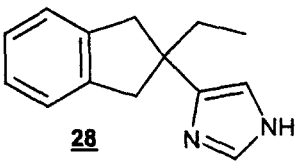

28

Conclusion - A number of potential pharmacological approaches to the management of ED are becoming available. However, to date the only approved oral therapy for the treatment of erectile dysfunction involves the selective inhibition of the type 5 phosphodiesterase. Considerable effort continues to be directed towards the discovery of novel structural classes of PDE5 inhibitors for potential utility in the treatment of ED as well as for other indications. A wide range of structural classes of PDE5 inhibitors are already known, although little is known about their progress in clinical development. Injectable formulations of vasodilators also show excellent efficacy in ED and efforts are being made to modify the methods of administration to ensure better tolerance and compliance.

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Chapter 9. Anticoagulants: Thrombin and Factor Xa Inhibitors

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Introduction - Thrombotic disorders remain the major cause of death and disability in western society and are projected to be the leading cause of death worldwide within twenty years. Conventional anticoagulant therapy for the treatment of acute and chronic thrombotic disorders has been built around the heparins and warfarin (Coumadin®) which are indirect inhibitors of the coagulation cascade. The heparins exert an antithrombotic effect by complexing with circulating serpin antithrombin III (ATIII) (1). Relative to heparin, low molecular weight heparins (LMWH) offer a favorable pharmacokinetic and pharmacodynamic profile and a higher factor Xa/thrombin activity ratio. However, as with heparin, clot bound thrombin (IIa) may not be accessible to inhibition and the parenteral route of administration remains an issue for extended treatment intervals (2). Coumadin®, a vitamin K antagonist which inhibits post translational γ-carboxylation of several coagulation zymogens, is the most widely prescribed oral anticoagulant and is being utilized as therapy in a growing number of chronic antithrombotic indications (3). However, both heparin and warfarin require careful monitoring of clotting times to achieve desired efficacy and dose titration to minimize excessive bleeding and potential drug interactions (4). The improved understanding of the coagulation process and the large window of opportunity associated with finding agents with simplified dosing, less rigorous monitoring, superior safety and comparable efficacy has provided the impetus for the search for new orally active anticoagulants to augment or replace existing therapies.

Thrombin, a serine protease of the trypsin family, plays a central role in thrombosis and haemostasis (5). It is the terminal enzyme in the coagulation cascade and is directly responsible for the cleavage of fibrinogen, resulting in the precipitation of insoluble fibrin, the primary component of a blood clot or thrombus. In addition, thrombin is key to the activation of platelets which stimulates aggregation. The development of selective, orally active, low molecular weight thrombin inhibitors has been an intense research focus in the search for new anticoagulants for a large number of pharmaceutical laboratories (6-14). At this time, the development of direct thrombin inhibitors is more advanced than other anticoagulant approaches. However, inhibition of thrombin generation by direct inhibition of factor Xa has recently emerged as an attractive target for the discovery of novel antithrombotic agents (9,12-14). Factor Xa (fXa), a trypsin-like serine protease similar in structure to other procoagulant proteases, plays a critical role in the coagulation cascade, serving as the point of convergence of the intrinsic and extrinsic activation mechanisms in the final common pathway of coagulation. FXa binds to phospholipid membranes, primarily to phosphatidyl serine and, together with factor Va and calcium ions, forms the prothrombinase complex which is responsible for the generation of thrombin *via* proteolysis of prothrombin. Since fXa inhibitors affect coagulation specifically but not platelet function, this mechanism may show decreased bleeding tendency relative to thrombin inhibitors and antiplatelet agents. This chapter will focus on the progress made over the past year toward discovering orally active thrombin and fXa inhibitors.

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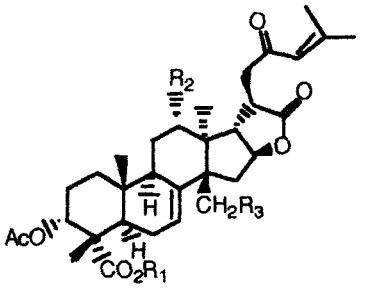
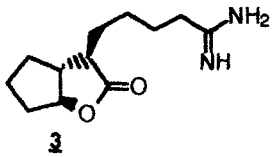
THROMBIN INHIBITORS

Naturally Occurring Thrombin Inhibitors - Thrombin inhibitors continue to be discovered in a variety of natural sources. Triterpenes **1** (GR133686) and **2** (GR133487), isolated from methanolic extracts of the leaves of the plant *Lantana camara L.* (wild sage), are tight binding, reversible competitive inhibitors of human α-thrombin (15,16). The mechanism of action of **1** and **2** is reversible acylation of thrombin on Ser195 by ring opening of the 5,5-*trans*-fused lactone moiety, followed by regeneration of the lactone. Synthesis of related 5,5-*trans*-fused lactones (e.g. **3**) has resulted in novel acylating inhibitors, however, unlike their natural product analogs, they are unstable in plasma (17). Two additional serine protease inhibitors, **4** (cyclotheonamide E2) and **5** (cyclotheonamide E3) have been isolated from the marine sponge *Theonella swinhoei*. Both **4** and **5** are closely related to cyclotheonamide E, differing only in the N-acyl group of the alanyl side chain (18). Another naturally occurring thrombin inhibitor published during the past year is Bothroalternin, a bothrojaracin-like protein, isolated from *Bothrops alternatus* venom (19).



1; $R_1 = R_2 = R_3 = H$
2; R_1 = β-D-Glucose, $R_2 = R_3 = OH$



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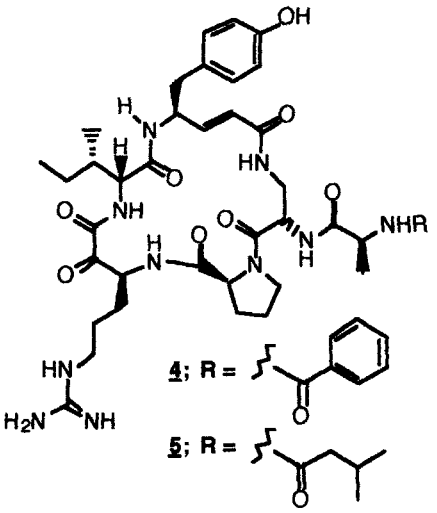


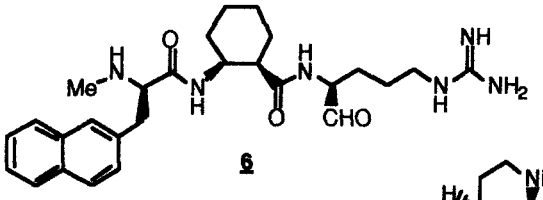
4; R =

5; R =

Electrophilic Transition State Inhibitors - Early thrombin inhibitors of this class employed a tripeptide sequence containing an electrophilic moiety capable of interacting covalently with the active site Ser195 hydroxy group to mimic a tetrahedral transition state in a reversible manner. While very potent inhibitors were discovered using this strategy (e.g., DuP 714 and efegatran amongst others), the issues of slow tight-binding kinetics, short plasma half-lives, poor oral bioavailability and poor selectivity versus other serine proteases, particularly trypsin-like enzymes unrelated to coagulation, were all of major concern. Several recent strategies have been employed to address these issues, including increasing the rigidity and/or hydrophobicity of the P1 group, reducing the basicity of the P1 group, and introducing nonpeptidic fragments into the P2 and P3-P4 regions (6-11,13,14). In arginal **6** (IIa $IC_{50} = 22$ nM), which is twice as potent and more selective than efegatran (Me-D-Phe-Pro-Arg-H; IIa $IC_{50} = 46$ nM), the proline P2 residue of efegatran was replaced with a ring constrained unit (a 1,2-disubstituted cyclohexane moiety) and the phenylalanine unit was replaced with a larger β-(2-napthyl)alanine (20). A series of peptide arginals

in which hydrophobic residues were introduced onto the nitrogen of the proline residue led to **7**, which also showed increased selectivity and oral bioavailability relative to efegatran (21).

A series of β,β-dialkylphenethylglycine P2 analogs of boropeptide DuP 714 (Ac-D-Phe-Pro-Arg-B(OH)$_2$), exemplified by **8** (IIa K_i = 0.06 nM, factor I IC_{50}/IIa K_i = 9000, compared with factor I IC_{50}/IIa K_i = 250 for DuP 714) were described having increased selectivity for thrombin over complement factor I relative to DuP 714 (22). The resulting inhibitors had less tendency to cause the side effects such as hypotension and elevated serum transaminases observed with DuP 714, which are believed to be mediated by inhibition of factor I. Replacement of the central proline of DuP 714 with an *N*-cyclopentylglycine residue afforded **9** (S18326), currently in Phase I clinical trials, which also has an improved selectivity profile (IIa IC_{50} = 0.50 nM, factor I IC_{50}/IIa IC_{50} = 21.1, compared to IIa IC_{50} = 0.55 nM, factor I IC_{50}/IIa IC_{50} = 2.9 for DuP 714) (23). More recently, S31214 (structure not published) showed a further enhanced selectivity profile for thrombin relative to factor I (24). Neither **9** nor S31214 caused hypotension in the rat or thrombocytopenia in the dog. Also in the boronic acid series, heterocyclic *cis*-amide mimics including pyrazole, 1,2,4-triazole and tetrazole have been shown to be effective heterocyclic replacements for the P2 residue (25).



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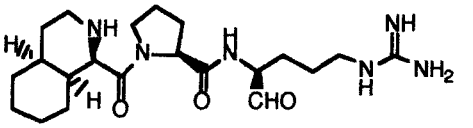

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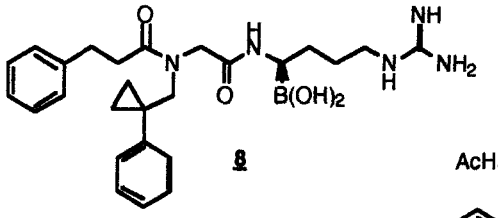

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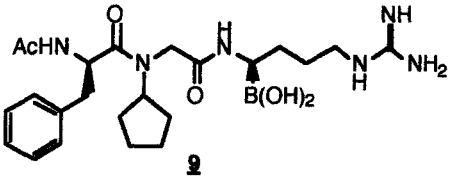

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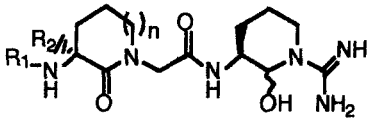
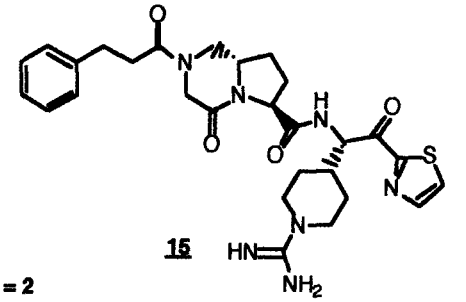


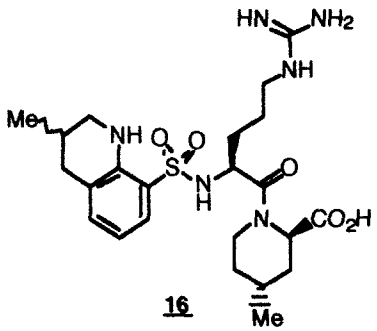
10; R_1 = $BnSO_2$, R_2 = H, n=1
11; R_1 = (2-MeO_2C)$BnSO_2$, R_2 = H, n = 2
12; R_1 = H, R_2 = Bn, n = 1
13; R_1 = $BnSO_2$, R_2 = CO_2Et, n = 1
14; R_1 = (2-$ChxCH_2O_2C$)$BnSO_2$, R_2 = H, n = 2



15

In the arginal series, lactam sulfonamides **10** (CVS 1578), **11** (CVS 2097) and quaternary amino derivative **12** (CVS 1897), in which the lactam serves as a Phe-Pro dipeptide mimic, were previously reported as potent transition state inhibitors (IIa IC_{50} = 6.2 nM, 0.75 nM and 2.05 nM, respectively) with good selectivity and oral bioavailability (26,27). Additional related peptidomimetic arginals including P3 lactams containing an ester moiety at the quaternary center, hydrophobic P3-P4 aminoalkyllactams and P4 alkoxycarbonylbenzylsulfonamides (e.g., **13** and **14**; IIa IC_{50} = 1.1 nM, and 0.56 nM, respectively) have recently been described (27-29). Use of a bicyclic template to further constrain the P2 region in combination with a variety of P1 serine trap modifications has led to several series of inhibitors (30-32). Ketothiazole **15** (IIa IC_{50} = 1 nM, trypsin IC_{50} = 11,940 nM) is an example from this class of compounds, and although good potency, selectivity, and antithrombotic efficacy has been achieved, significant oral bioavailability remains an issue (30).

Non-Covalent Inhibitors Based on Argatroban, NAPAP and TAPAP - Non-covalent or non-electrophilic thrombin inhibitors are compounds which do not bind covalently with Ser195 but which form strong hydrophobic and electrostatic interactions with the enzyme. Prototypical low molecular weight non-covalent thrombin inhibitors include compounds such as argatroban, NAPAP and TAPAP which contain highly basic, polar functionality and are not orally active. Argatroban (**16**), an intravenous anticoagulant, has been approved in Japan for the treatment of peripheral arterial occlusive disease and acute ischemic stroke and remains the only marketed low molecular weight thrombin inhibitor. In structurally related compound **17** (UK-156,406), currently in Phase II clinical trials, benzamidine was successfully employed as a P1 arginine surrogate to give rise to an orally bioavailable inhibitor. Compound **17** has a K_i against thrombin of 0.46 nM and is approximately 50-fold selective for thrombin compared to trypsin (K_i = 26 nM) (33). In dogs, **17** shows good oral bioavailability (F = 45%), but has a relatively short plasma half-life of 0.8 hours. In



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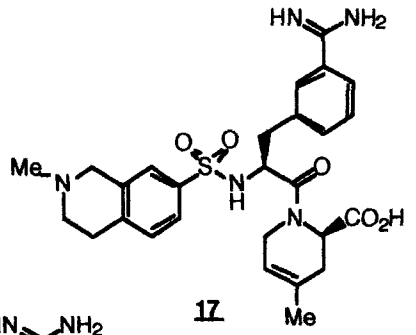

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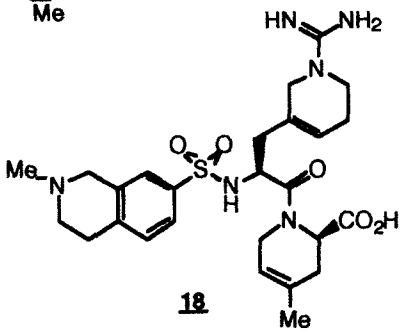
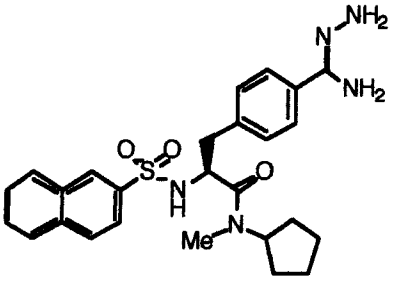


18

clinical studies, **17**, when given at single oral doses of 10, 25, 50, 100 and 200 mg, was well tolerated and at the 100 and 200 mg doses both TT and APTT elevations were maintained (10X control for TT and 1.2X control for APTT) for 4 hours (33). Recently, **18** (UK-239,326; IIa K_i = 0.06 nM, and trypsin K_i = 50 nM) has been reported as a back-up candidate for **17** having improved potency and selectivity (34).

As it is generally accepted that highly basic moieties are barriers for oral absorption, a number of synthetic strategies have been utilized to improve oral absorption by replacing the basic P1 residue with a less basic side chain. Employing the less basic benzamidrazone residue (pKa ~8.9) at the P1 position in a TAPAP-like series provided the *N,N*-cyclopentylmethylamine derivative **19** (LB30057) as a clinical candidate which is potent (IIa K_i = 0.38 nM), selective (trypsin K_i/IIa K_i = 8,658) and orally bioavailable (58% in dogs with a mean elimination half-life of 182 min) (35). Efforts to further enhance the thrombin inhibitory potency of this series by replacing the naphthyl moiety of the sulfonamide with a variety of hydrophobic moieties led to more potent analogs such as **20** (IIa K_i = 0.045 nM, trypsin K_i/IIa K_i = 12,222); however, relatively low oral absorption was observed (36). Both 2,5-thienylamidine (e.g, **21**; IIa K_i = 3.0 nM) and *p*-benzylamine (e.g., **22**; IIa K_i = 18.3 nM), the latter of which was developed *via* a combinatorial approach, have been incorporated as P1 surrogates in this series (37-40). Compound **23** (CGH1668; IIa K_i = 39 nM) bearing an aminophenylalanine as the P1 moiety is another example derived from **16** which contains a weakly basic P1 replacement (41). Chemical optimization of **23** afforded **24** (IIa K_i = 22 nM) which demonstrated improved pharmacokinetics in rats (F = 61% after p.o. administration at 10 mg/kg, elimination half-life of 59 min following intravenous dosing at 3 mg/kg) relative to **23**.



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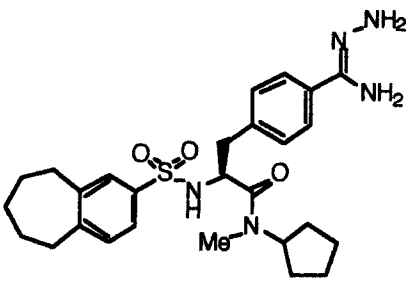

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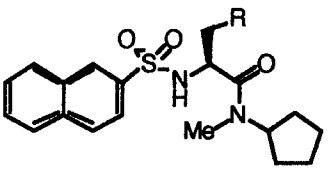
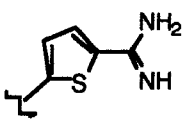
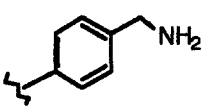
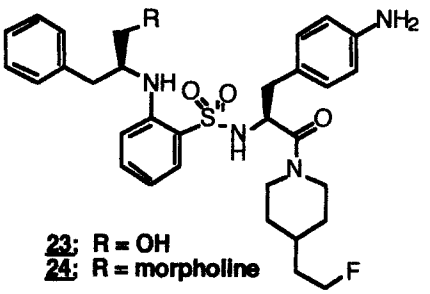


21; R =



22; R =

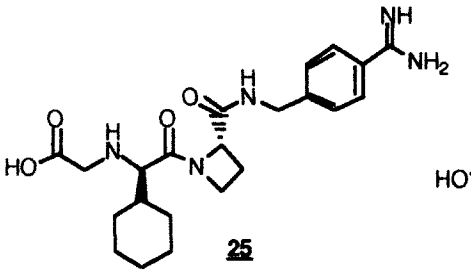




23; R = OH
24; R = morpholine

Non-Covalent Inhibitors Derived from Peptidomimetics - Currently, the most clinically advanced orally active non-covalent thrombin inhibitor is **25** (melagatran). Melagatran, a benzamidine analog of inogatran designed to mimic fibrinopeptide A, has been shown to be efficacious in animal models of thrombosis (42, 43). Acylguanidine **26** (pKa = 7.6), derived *via* an effort to reduce the basicity of the P1 guanidine in an inogatran analog, showed only marginally decreased *in vitro* activity compared to the corresponding guanidine despite being five orders of magnitude less basic (IIa IC_{50} 240 nM compared to 110 nM) (44).

Another potent and selective non-covalent thrombin inhibitor, cyclohexylamine **27** (L-371,912), was previously reported to result from the discovery that the α-ketoamide serine trap from related inhibitors could be removed with only a 50-fold loss in potency (45). A resin-based rapid synthesis approach to evaluate replacement of the P3 residue with lipophilic carboxylic amides in an attempt to improve the oral activity of **27** led to hydroxyfluorenyl carboxamide **28** (L-372,460; IIa K_i = 1.5 nM) which was both orally bioavailable in dogs and monkeys and efficacious in the rat ferric chloride-induced thrombosis model (46). In this series, solid phase synthesis was also utilized to explore novel lipophilic P1 moieties of reduced basicity to try to achieve higher oral bioavailability. These efforts resulted in potent thrombin inhibitors bearing neutral P1 groups, the most potent of which was the 2,5-dichloro analog, **29** (IIa K_i = 3 nM) (47). Although **29** exhibited good oral bioavailability (F = 45%), high protein binding resulted in unacceptable efficacy in the rat ferric chloride-induced thrombosis model. Oxyacetic amide **30** (IIa K_i = 0.74 nM), with the smaller less lipophilic D-cyclohexylglycine at the P3 position and an *N*-ethyl amide on the 2-position of the P1 aromatic ring, had the best overall profile, being efficacious and orally bioavailable in rats, dogs and cynomolgus monkeys but its short half-life precluded



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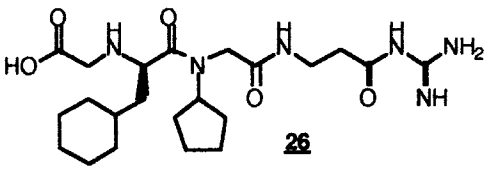

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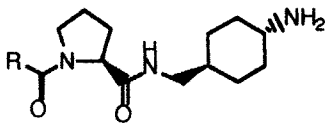

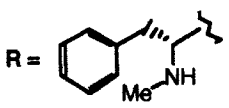

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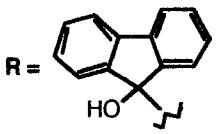

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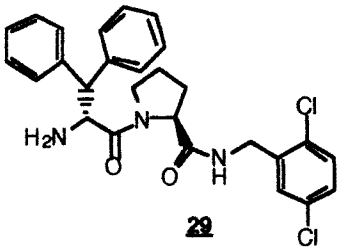

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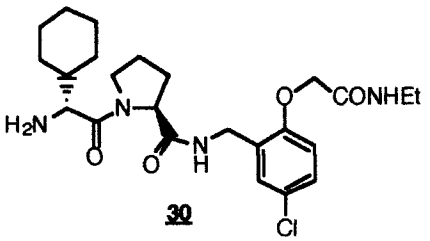
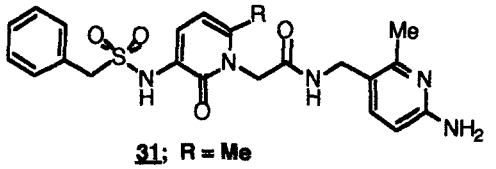


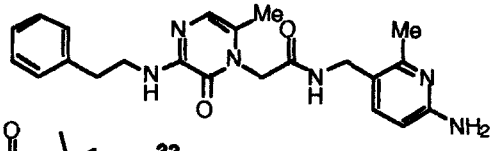
30

clinical development (48). The X-ray crystal structures of **29** and **30** bound to thrombin show a novel binding mode in the S1 pocket. In both compounds the 5-chloro substituent makes a direct lipophilic contact with the aromatic ring of Tyr228. Additionally, the oxyacetic amide side chain of **30** contributes additional binding energy by participating in a hydrogen bonding interaction with the N-H of Gly219 (48).

Incorporating a pyridinone acetamide peptidomimetic template in conjunction with the less basic 2-amino-6-methyl pyridine led to **31** (L-374,087) which is highly selective (IIa K_i = 0.5 nM, trypsin K_i = 3200 nM), efficacious in the rat ferric chloride model and orally bioavailable in dogs (F = 44%) with a 2.5 hour half-life after intravenous infusion (49). Substitution of the C6 pyridinone with an *n*-propyl group in place of the methyl group resulted in **32** (L-375,052), which retained the potency, selectivity and antithrombotic efficacy of **31** and showed improved oral absorption in rats and dogs (50). Replacement of the pyridinone core with the chemically stable pyrazinone bioisostere afforded **33** (L-375,378). This compound was selected for clinical development based on its comparable potency, selectivity (IIa K_i = 0.8 nM, trypsin K_i = 1800 nM) and efficacy in the rat ferric chloride model and improved pharmacokinetics (F = 95%, $t_{1/2}$ = 231 min. in dogs and F = 60% and $t_{1/2}$ = 49 min. in rhesus monkeys) relative to **31** (51). Imidazole P1 groups have been reported in several non-covalent series, one example is **34** (L-376,062; IIa K_i = 0.36 nM) (52-54). The 1-aminoisoquinoline moiety (pKa = 7.5) has also been used as a less basic P1 moiety (55). In one recent example, replacement of the highly basic benzamidine moiety of NAPAP by 1-aminoisoquinoline resulted in **35** (Org 37476; IIa IC_{50} = 82 nM) which showed enhanced Caco-2 cell permeability and improved trypsin selectivity (56).



31; R = Me
32; R = n-Pr



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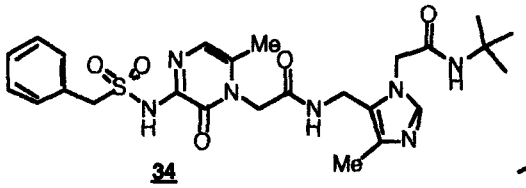

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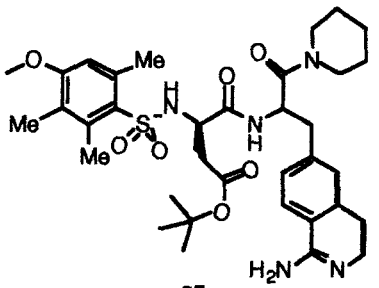

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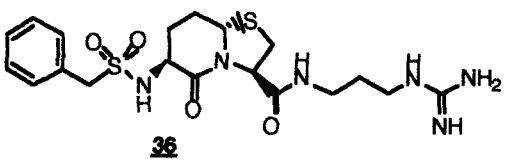

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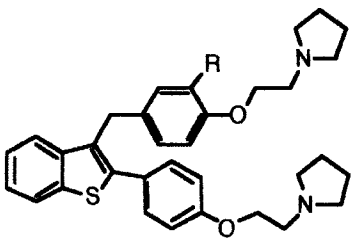
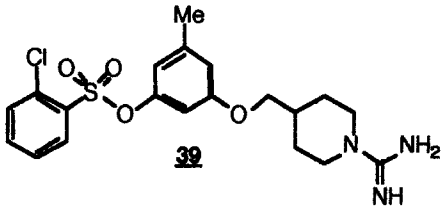
Another departure from the tripeptide motif, while less potent, incorporated a 7-thiaindolizidinone bicyclic core (**36**; IIa K_i = 111 nM) in the S2 region to reduce the flexibility of the ligand (57).

Non-peptide Derived Inhibitors - Compounds **37-42** exemplify recently disclosed non-peptidic reversible competitive thrombin inhibitors. The potency of a series of benzo[b]thiophenes has been increased by placing small hydrophobic groups, such as Br and CH_3, at the C-3" position of the C-3 aryl ring, thereby increasing interactions in the S2 binding site (**37** and **38**) (58). X-ray crystallography has shown that the benzo[b]thiophenes bind to thrombin in a novel manner, with the benzothiophene in the S1 specificity pocket and the C3 aryl ring near the opening of the S2 pocket defined by residues Trp86, Leu132, His79 and Tyr83. Additionally, a series of related amino- and aminocyanopyrido[b]thiophenes has been described (59).

A second series lacking an amide backbone, which was derived using a 1,3,5-trisubstituted phenyl ring as the central scaffold has also been disclosed (60-64). Compounds **39** and **40** (IIa K_i = 4.6 nM and 13 nM, respectively) and related compounds are anchored to the enzyme *via* a single network of hydrogen bonds to Asp189 of the S1 pocket (60, 61). Aminopyridine **42** (IIa K_i = 1.3 μM) was the result of modification of **41** (L-636,619; IIa K_i = 0.7 μM) which was identified *via* topological similarity searching over the Merck Corporate Sample Database (65). Another series of 4-aminopyridines has recently been described in the patent literature (66).



37; **R = Br**
38; **R = Me**



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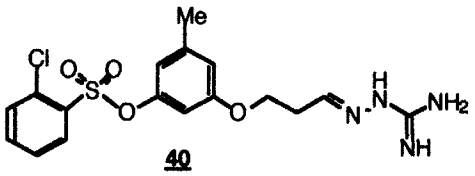

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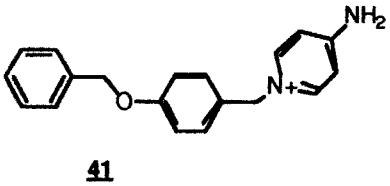

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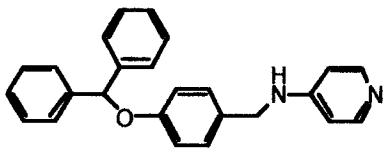


42

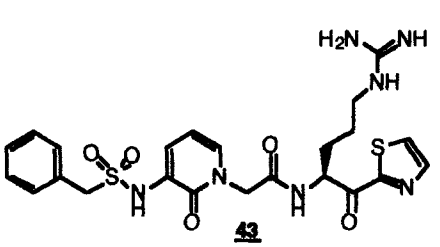
FACTOR Xa INHIBITORS

Although not as advanced as the direct thrombin inhibitor area, the inhibition of thrombin generation by the direct inhibition of fXa is clearly emerging as an important alternative strategy in the search for new and effective antithrombotic agents. The established antithrombotic effects of very potent and selective naturally occurring

fXa inhibitors such as tick anticoagulant peptide (TAP), antistasin (ATS) and nematode anticoagulant protein-5 (NAP-5) have provided validation for the inhibition of fXa as an antithrombotic strategy (67-69). Other approaches to fXa inhibition have been directed at synthetic pentasaccharides, electrophilic transition state analogs and dibasic non-covalent inhibitors. In general, these approaches have been limited by low oral bioavailability. Recently, considerable progress has been realized in the discovery of monobasic non-covalent inhibitors. Clearly, the research focus in many companies is on finding small molecule non-covalent fXa inhibitors with reduced basic character, which are expected to have a greater chance of being suitable for oral dosing. Several small molecule fXa inhibitors representing the above approaches have demonstrated promising results in various antithrombotic models. Ultimately, clinical results are needed to determine if direct fXa inhibition has efficacy and/or safety advantages relative to direct thrombin inhibition or to existing therapies.

Synthetic Pentasaccharides - SR 90107/Org 31540 is a sulfated synthetic pentasaccharide which represents the minimum sequence of the heparin chain which interacts with antithrombin III (ATIII). This compound, currently in Phase III clinical trials for deep vein thrombosis and arterial thrombosis, selectively enhances the ATIII-dependent inhibition of fXa and has been shown to be an effective antithrombotic agent when administered subcutaneously (70). SANORG 34006 is a "non-glycosaminoglycan" pentasaccharide analog which has improved affinity for ATIII and favorable pharmacokinetics relative to SR 90107/ Org 31540 (71). SANORG 34006 did not increase bleeding in rabbits at a dose tenfold higher than the antithrombotic ED_{50} in this species, representing a more favorable therapeutic index than that of heparin (71).

Electrophilic Transition State Inhibitors - A series of patent applications has recently published in which an electrophilic P1 arginine residue is combined with various peptidomimetic templates (72-79). An example from this class of compounds is the arginine ketothiazole **43**, which contains a pyridinone peptidomimetic which has been applied to the thrombin inhibitor area (49,80). Compound **43** has a fXa IC_{50} of 3 nM but is not selective over thrombin (IIa IC_{50} = 6 nM) (72). The ketoamide **44** has been disclosed, with a fXa K_i of 54 nM. The diarylsulfide P3 residue in **44** was reported to contribute to improved fXa affinity and selectivity over thrombin in corresponding borolysine analogs (81).



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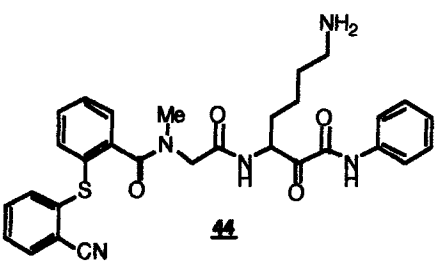

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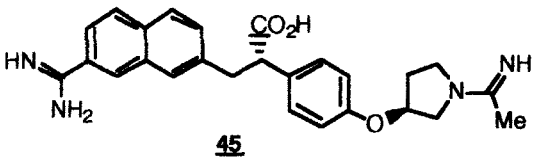
Dibasic Non-Covalent Inhibitors - This class of inhibitors is generally characterized by a *meta*-substituted benzamidine which interacts with Asp189 in the S1 pocket and an additional basic group which binds in the S4 aryl binding pocket. The dibasic compound **45** (DX-9065a; fXa K_i = 41 nM) was the first example of a potent, small molecule fXa inhibitor which was highly selective over thrombin (K_i > 2000 μM) (82). Compound **45** was found to be an effective antithrombotic agent which has advanced

into clinical trials. Intravenous administration to healthy human volunteers demonstrated that **45** was well-tolerated, exhibited dose-reponse anticoagulation as measured by activated partial thromboplastin time (aPTT) and had no bleeding propensity (83). However, its bioavailability is reported to be low in rats and baboons (84,85).

Compound **45** has served as a template for the design of related inhibitors. Modification of the acid moiety led to the more potent **46a** (YM-60828; fXa K_i = 1.3 nM), which has been studied in a variety of animal thrombosis models (86-88). In rat thrombosis models, **46a** was reported to be superior to argatroban, heparin and dalteparin in terms of the bleeding risk-antithrombotic benefit ratio (89). Its mesylate salt, **46b** (YM-75466), gave similar results when compared to warfarin in a rat venous thrombosis model (90). Compound **46a** was found to be 20% bioavailable in squirrel monkeys (91). Phosphonate analogs such as **47** have appeared in the patent literature (92). Tetrahydroisoquinoline-based P1 analogs, such as **48**, are claimed to be more readily synthesized than **45** and have similar potency (fXa IC_{50} = 55 nM) (93). Other patent disclosures claim biphenylamidines such as **49** (FX-2212a) and **50** (fXa



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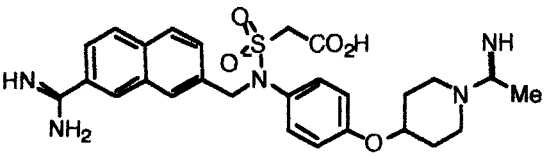
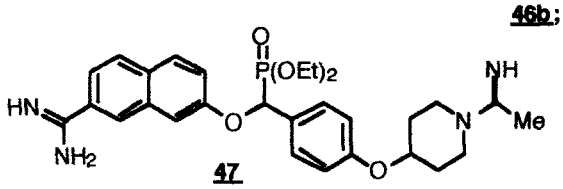


46a; **bis HCl salt**
46b; **monomesylate salt**



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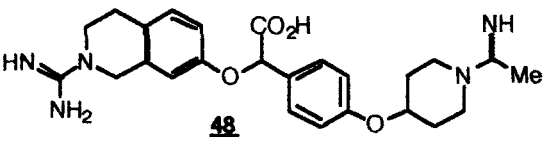

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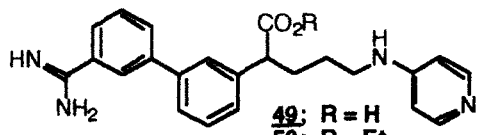
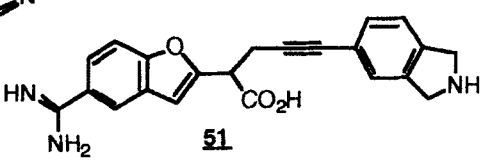


49; **R = H**
50; **R = Et**



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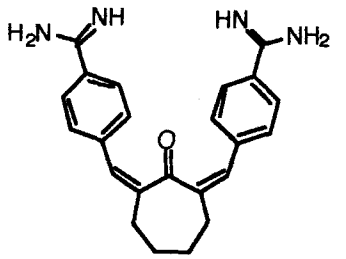
IC_{50} = 250 nM and 69 nM, respectively) and benzofuran amidines such as **51**, which has a fXa IC_{50} of 230 nM (94,95). The X-ray crystal structure of the carboxylic acid **49** bound in the active site of des[1-44] fXa has been solved (96). Interestingly, two different crystal forms are observed, each of which only contains the *S*-isomer of **49**. In both crystal forms the biphenyl amidine resides in the S1 specificity pocket while the pyridine moiety binds in the S4 aryl binding pocket.

Another potent series of dibasic fXa inhibitors is based on an analysis of **52** ((*Z,Z*)-BABCH; fXa K_i = 0.66 nM), which was found to be 300-fold and 25,000-fold more potent toward fXa than (*E,Z*)-BABCH and (*E,E*)-BABCH, respectively (97). A bis-amidino-2,6-diphenoxypyridine template was designed to mimic the favored conformation of **52**, leading to compound **53** with a K_i of 13 nM (98). Optimization led to **54** (ZK-807191 or BX-807834), where the P1 hydroxy group as well as the sarcosine substitution on the pyridine core led to significant enhancement of potency. Compound **54** was chosen for clinical development on the basis of its potency (fXa K_i = 0.1 nM), selectivity (trypsin K_i = 280 nM, IIa K_i = 2000 nM) and pharmacokinetic profile (98). Interestingly, the basic imidazoline residue in this series



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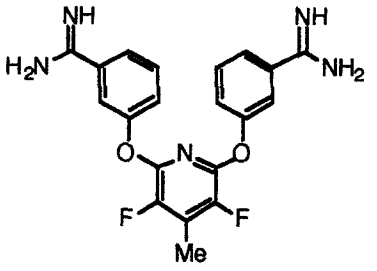

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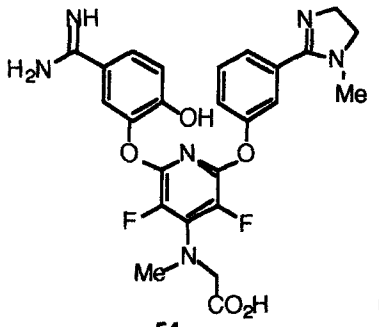

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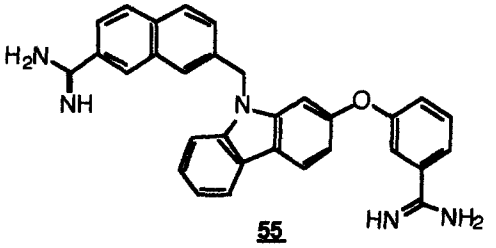

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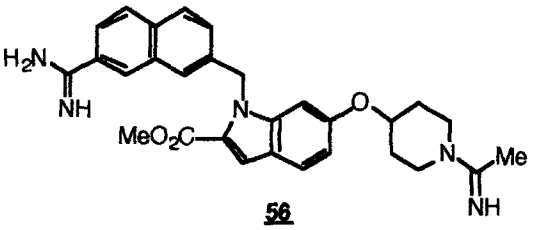

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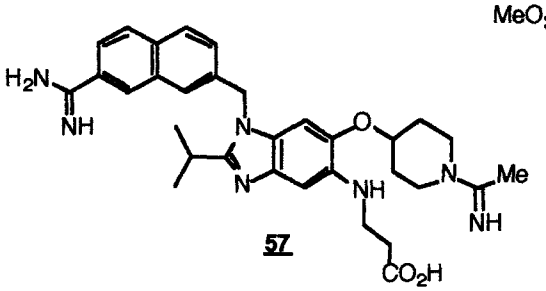
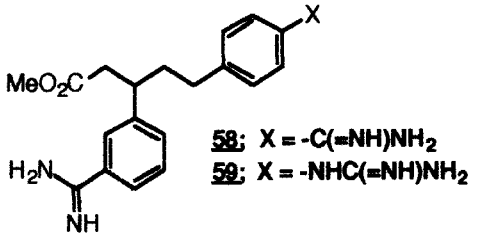


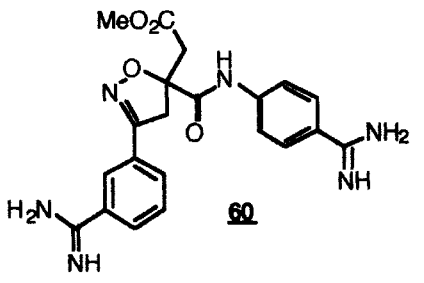
57

afforded compounds with more favorable pharmacokinetics relative to analogs containing the neutral *N,N*-dimethyl amide residue. In dogs and baboons, **54** was found to have an oral bioavailability of about 20%, but it had a fairly short plasma half life ($t_{1/2}$ ~ 1-2 h). In both species bioavailability was significantly decreased by prior administration of food (99). In a rat venous stasis model, **54** effectively inhibited thrombus formation and had a favorable therapeutic ratio (bleeding ED_{50}/efficacy ED_{50}) relative to rTAP or warfarin in the same model (100). Several related series of inhibitors were designed to mimic the favored conformation of **52** (101-104). Examples of these approaches are represented by the carbazole analog **55** (fXa K_i = 0.30 nM), the indole analog **56** (fXa K_i = 0.17 nM) and the very potent benzimidazole **57** (fXa K_i = 0.01 nM, trypsin K_i = 3.5 nM) (101-103). Structurally, the naphthylamidines **55**, **56** and **57** are closely related to the previously discussed naphthylamidines **45**, **46a** and **46b**.

Other examples of dibasic inhibitors include the bis-amidine **58** (fXa K_i = 34 nM) and the corresponding guanidine analog **59** (fXa K_i = 9 nM), which were designed using molecular modeling studies based on the des[1-44] fXa crystal structure (105). A series of isoxazoline-based bis-amidines has also been disclosed, as represented by **60**, which has a K_i of 94 nM (106). A series of bis-amidines represented by **61** was recently described (107). The 4,4'-bisamidino isomer shown was found to be more potent than the corresponding 3,4'-bisamidino isomer derived from 3-amidinophenylalanine, contrary to what is generally observed. A crystal structure of **61** bound to trypsin showed an inverted binding mode, wherein the 4'-amidinobenzenesulfonyl moiety unexpectedly binds in the S1 pocket while the 4-amidinophenylalanine binds in the S4 aryl binding pocket. The bisamidine **62** inhibits fXa with a K_i of 10 μM in the presence of EDTA (absence of zinc) but in the presence of added



58; X = -C(=NH)NH$_2$
59; X = -NHC(=NH)NH$_2$



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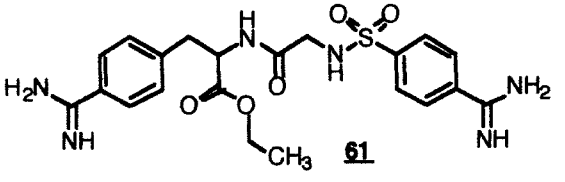

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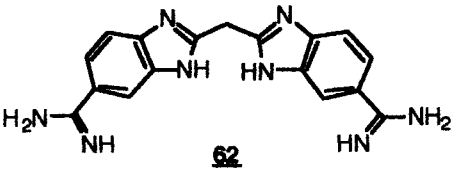
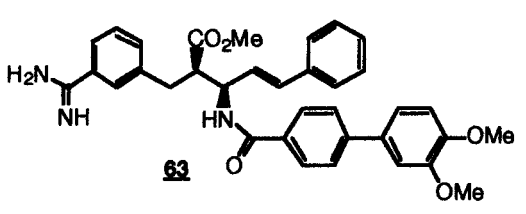


62

Zn^{2+} its K_i value is 0.001 μM (108). This zinc-dependent potency increase can be rationalized by a binding mode in which Zn^{2+} is tetrahedrally coordinated with the two chelating benzimidazole nitrogens and two active site residues, His57 and Ser195. However, **62** is also a potent inhibitor of the related serine proteases trypsin, thrombin and tryptase.

Monobasic Non-Covalent Inhibitors - Most of the effort in this class of inhibitors has been directed at replacing the P4 basic residue with a hydrophobic group while maintaining the P1 *meta*-benzamidine residue, which appears to be optimal for fXa potency. A series of 2,3-disubstituted β-alanines with a biphenyl P4 group has been reported as represented by **63** (*cis*-racemate) with an IC_{50} of 0.03 μM (109). The purine analog **64** (fXa K_i = 0.42 nM) has been reported in which an *N,N*-dimethyl benzamide resides in the S4 pocket (110). This compound is an analog of the diphenoxypyridine **54**, which also retains significant fXa potency when the P4 imidazoline is replaced by an *N,N*-dimethyl amide residue (98). A solid-phase approach to related monobasic amidinophenoxypyridine inhibitors has also been described (111). Molecular modeling was used to design a pyrrolidinone template, which led to a potent series of inhibitors such as **65** (RPR-120844; fXa K_i = 7 nM, 80-fold selectivity over trypsin) which contains an amidinothiophene P1 group with a naphthyl P4 group (112). Modifications of P1 and P4 led to **66** (RPR-130737; fXa K_i = 2 nM), with improved potency and >1000-fold selectivity over trypsin. In a rabbit venous thrombosis model, **65** was found to be highly effective although it had a short duration of action (113).



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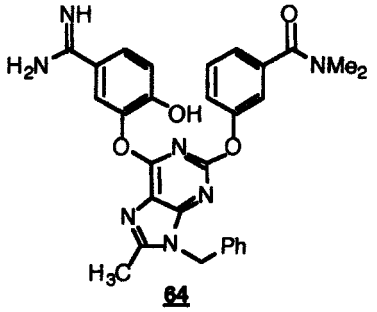

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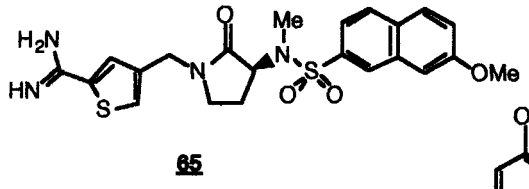

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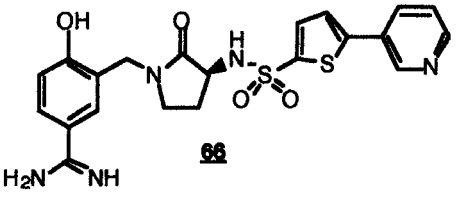

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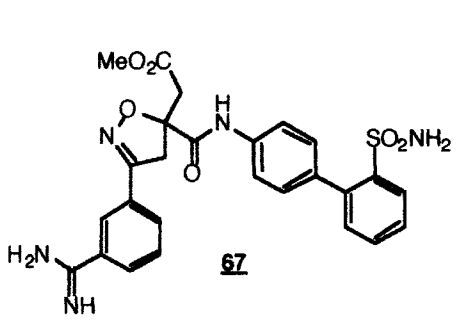
Several reports have shown that hydrophobic biphenylsulfonamide and related moieties appears to be very well-suited for binding in the S4 aryl binding pocket of fXa. The isoxazolines **67** and **68** (SM084) (fXa K_i = 6.3 nM and 0.11 nM, respectively) are examples utilizing this substitution which have improved fXa affinity relative to their P4 benzamidine counterparts (i.e., **60**) (114). The tetrazole **68** was found to be highly potent in a rabbit arterio-venous (A-V) shunt thrombosis model (ID_{50} = 0.032 μmol/kg/h). A similar substitution in the acyclic-linked series (i.e., **58**) led to compound **69**, a potent (fXa K_i = 0.53 nM) and achiral inhibitor (115). Additional reports have described the use of 5-membered aromatic heterocycles as core

templates with vicinal substitution of the P1 benzamidine and P4 biphenylsulfonamide groups. The isoxazole **70**, with a K_i of 0.15 nM, was the first reported example using this template (116). Replacement of the isoxazole ring with a pyrazole led to **71** (SN429; fXa K_i = 0.013 nM, trypsin K_i = 16 nM), which had enhanced potency relative to **70** (117). The pyrimidine analog **72** (SN116; fXa K_i = 0.041 nM, trypsin K_i = 150 nM) maintained high potency and also had improved trypsin selectivity relative to **71**. The pyrimidine **72** was also found to have an improved pharmacokinetic profile relative to **71**, with a 2-fold lower clearance and a 4-fold longer half-life after intravenous dosing in dogs. Both **71** and **72** were very potent in a rabbit arteriovenous (A-V) shunt thrombosis model (ID_{50} = 0.023 and 0.034 μmol/kg/h, respectively). Related compounds containing a 6-membered heterocyclic core template have appeared in the patent literature (118). Using a cycloheptylurea core template, sulfonylpiperidines were found to be effective P4 hydrophobic groups, as in **73** (fXa K_i = 10 nM) (119). Other examples of monobasic fXa inhibitor templates have appeared in the patent literature (120, 121).



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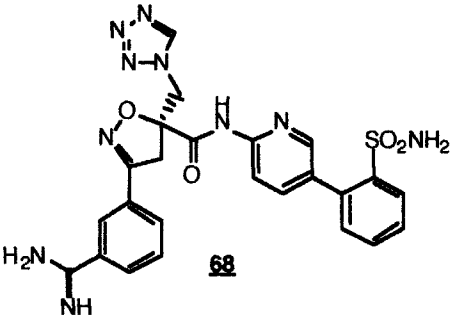

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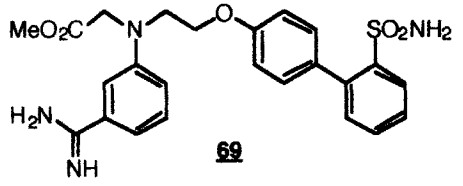

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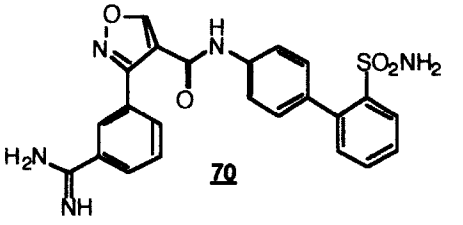

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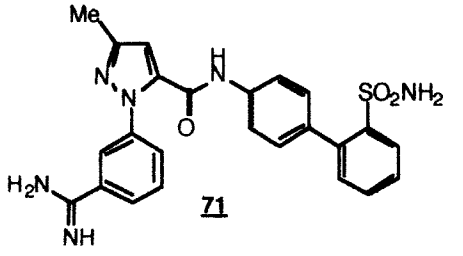

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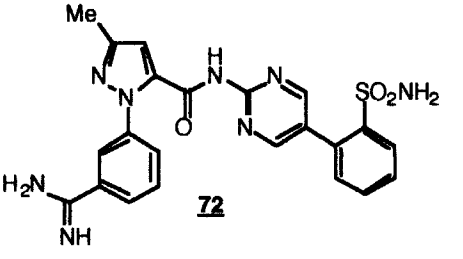

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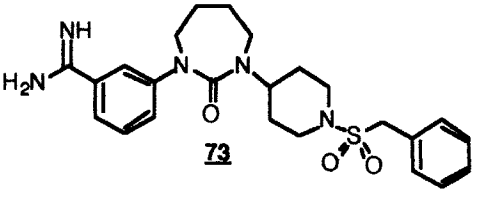

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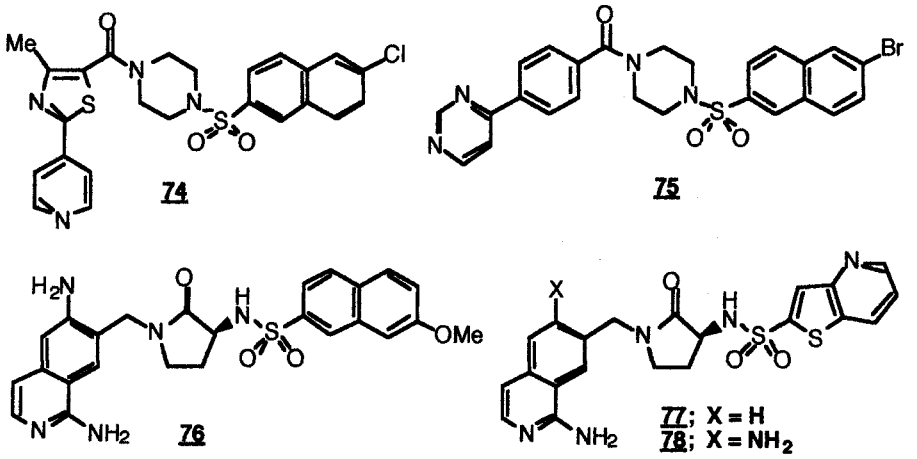
Less Basic Non-Covalent Inhibitors - Additional fXa inhibitors which substitute the P1 *meta*-benzamidine with less basic benzamidine mimics have appeared. A series of inhibitors containing a pyridine group in P1 has appeared in the patent literature, an example of which is compound **74**, with an IC_{50} of 0.019 μM (122). This patent is closely related to earlier patent disclosures (123-125). Another related patent disclosure includes less basic and neutral heterocycles as P1 groups, such as the pyrimidine analog **75**, although no binding data are reported (126). The 1-aminoisoquinoline moiety has been used as a less basic substitute for *meta*-benzamidine, as in compound **76** (fXa K_i = 80 nM), although the potency is reduced considerably relative to related amidines (i.e., **65** and **66**) (127). Modifications to the P4 residue led to the more potent inhibitors **77** and **78** (fXa K_i = 22 nM and 6 nM, respectively) (128). The 6-amino substituent of **76** and **78** appears to strongly contribute to the potency of this series. Significantly, **77** was reported to have an absolute bioavailability of 53% in dogs, compared to a related amidinothiophene P1 lactam analog (i.e., an analog of **65**) which had negligible bioavailability in the same study (128). The X-ray crystal structure of **77** bound to fXa has been solved, which is the first reported structure of a non-amidine:fXa complex. This structure reveals that the aminoisoquinoline moiety binds in the S1 pocket with the amino group engaged in a hydrogen bond with the carbonyl oxygen of Gly218 and the isoquinoline ring nitrogen participating in a water-mediated hydrogen bond with Asp189 (128). This binding mode is in contrast to the bidentate interaction with Asp189 that is observed with amidines (96). The thienopyridine moiety binds well in the S4 pocket, sandwiched between Tyr99 and Phe174. Additional patent applications have appeared which claim fXa inhibitors containing less basic benzamidine mimics and neutral groups as the P1 functionality, although no binding data are reported for these compounds (129-133).



Conclusions - Recently, tremendous progress has been made in the discovery of potent, orally active, selective thrombin inhibitors *via* identification of non-covalent inhibitors with less basic functionality replacing the guanidino group of the arginine or amidino group of the benzamidine moiety. Several of these inhibitors are currently in clinical trials. In the fXa arena within the past year, much progress has been made from the early dibasic templates such as DX-9065a, which suffer from low oral bioavailability. Again, the focus in this area is clearly evolving toward the use of less basic substituents in order to improve on absorption and pharmacokinetic

characteristics. Within the next couple of years, as orally active fXa inhibitors are advanced into clinical trials, it should be possible to clarify which class of compound will be most efficacious and/or will have lower bleeding risk in clinical settings for given thrombotic indications.

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Chapter 10. Atherosclerosis: Emerging Pharmacological Approaches

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Introduction - In the past decade there have been intensive educational programs aimed at informing the public about the dangers of coronary artery disease (CAD). These programs are epitomized by the Expert Panel of the National Cholesterol Education Program (NCEP)) in the USA and the International Task Force for the Prevention of Coronary Heart Disease in Europe. The NCEP panel has recognized 10 risk factors for the development of CAD, including smoking, hypertension, obesity, gender, diabetes mellitus and elevated low density lipoprotein-cholesterol (LDL-C) (1). However, only elevated LDL-C has been shown to be an independent risk factor for the development of CAD (2). In recent years, several clinical trials (4S, WOSCOPS, CARE) have demonstrated the beneficial effects of LDL-C lowering on both morbidity and mortality, however, these trials show that the clinical benefit derived from aggressive LDL-C lowering is limited. For example, in patients with pre-existing CAD (4S trial) treatment with the HMG-CoA reductase inhibitor (HMGRI), simvastatin, gave a 36% decrease in LDL-C that resulted in a 36% reduction in events and a 22% reduction in mortality (3). In the WOSCOPS trial, patients were selected with elevated LDL-C levels but no pre-existing CAD, treatment with pravastatin resulted in a 31% reduction in events and a 22% reduction in mortality (4). As can be seen from these results and despite attempts to modify secondary risk factors such as smoking, obesity and hypertension, only approximately 30% of patients receive any long term benefit from these therapies. Greater reductions in LDL with more effective drugs or drug combinations are expected to produce greater reductions in cardiovascular events. However, other abnormalities in lipoprotein metabolism also contribute to the development of CAD, these include hypertriglyceridemia (elevated levels of triglycerides), hypoalphalipoproteinemia (low levels of high density lipoprotein-cholesterol (HDL-C)), mixed hyperlipidemias and elevated levels of lipoprotein (a) (Lp(a)). These abnormalities have now come to the forefront of research in many industrial and academic institutions. In addition to finding new therapies for these abnormalities, many of these institutions have turned their attention to influencing events directly at the artery wall.

This article will describe recent developments in the search for agents to correct some of the more commonly encountered lipoprotein abnormalities. In addition, we will focus on recent efforts to understand the pathogenesis of atherosclerotic lesions and to develop an understanding of lesion vascular biology with the aim of designing new therapies acting directly at the site of the disease, the artery wall.

LIPOPROTEIN TARGETS

Low Density Lipoproteins (LDL) -The success of the statins and the growth of the cholesterol-lowering market have encouraged further research into other mechanisms of LDL reduction. Although examination of other enzymatic steps in the cholesterol biosynthetic pathway have not yielded products competitive with the HMGRI's, efforts to inhibit the secretion of apoB-containing lipoproteins by inhibiting lipid transfer to apoB during VLDL assembly have resulted in the discovery of novel clinical candidates. This transfer is catalyzed by microsomal triglyceride transfer protein (MTP). MTP is required for apoB secretion, and it has been demonstrated in cell culture systems that decreases in apoB secretion are proportional to decreases in MTP-mediated transfer

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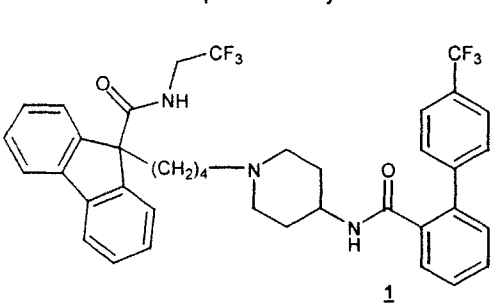
(5). The biphenyl carboxamide (1) inhibited MTP-mediated triglyceride transfer with an IC_{50} = 0.5 nM and normalized plasma lipids in Watanabe rabbits (6). It is unknown if lipid-lowering activity, comparable to the HMGRI's, can be obtained in humans without significant fat malabsorption or fatty liver.



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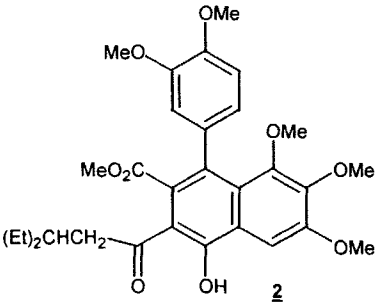

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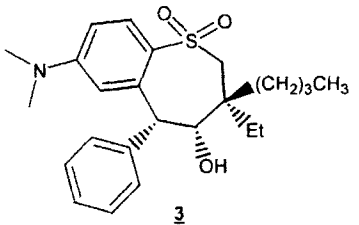

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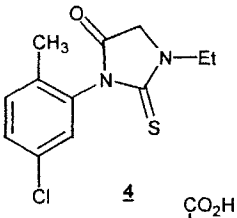

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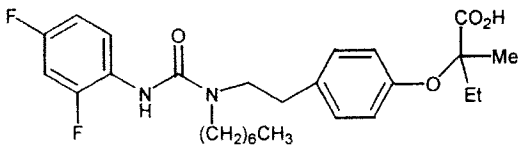

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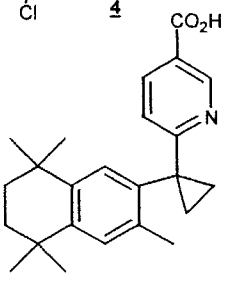

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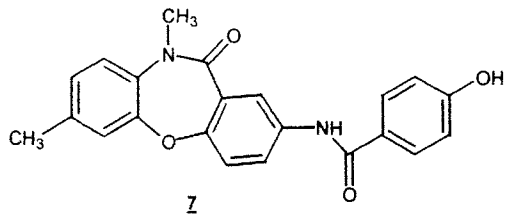

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Another target for achieving LDL reduction is inhibition of bile acid reabsorption. The active uptake of bile acids in the ileum requires a 99 kD protein called the ileal bile acid transporter (IBAT). A lignan derivative, S-8921 (2), not only inhibited uptake of bile acid in ileal vesicles, but also inhibited the uptake of radiolabeled taurocholate in COS7 cells expressing hamster IBAT (IC_{50}=66 μM) (7). It also lowered plasma cholesterol up to 50% at 22 mg/kg in chow-fed hamsters (8), and dose-dependently reduced plasma cholesterol and atherosclerosis in cholesterol-fed rabbits (9). Another IBAT inhibitor, SC-112 (3), possesses nanomolar (IC_{50}=5.6 nM) in vitro activity (10). It remains to be determined whether IBAT inhibitors can provide a degree of LDL reduction greater than resins (>20-25%), and some concern exists about the potential for bile salt-induced diarrhea.

High Density Lipoproteins (HDL) - Compound (4, WAY 135433) increased HDL-C in rats and monkeys primarily by stimulating the catabolism of triglyceride-rich lipoproteins (11). Activators of peroxisome proliferator activated receptors (PPAR's) can modestly

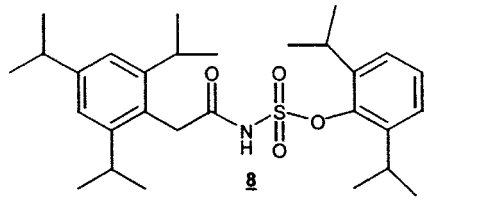
elevate HDL-C, and this subject has been reviewed recently (12). GW2331 (**5**), a novel fibrate, activates several PPAR's and also binds to them with high affinity (13). PPAR's bind to target DNA sequences as heterodimers with RXR, and certain synthetic RXR-selective ligands e.g. LG100268 (**6**) have been shown to have multiple effects including elevation of HDL-C (14).

Another potential approach for increasing HDL-C is the identification of compounds that induce the expression of lecithin:cholesterol acyltransferase (LCAT). Transgenic animal data strongly suggests that enhancing LCAT activity would increase HDL-C and reduce atherosclerosis (15). It has been suggested that inhibition of DGAT (diacylglycerol acyltransferase) would cause a shift in the flux of fatty-acyl CoA into phospholipid that would be secreted in HDL, thus making HDL a better acceptor of peripheral cell cholesterol. DGAT inhibitors, such as the benzoxazepinone (**7**) have been synthesized for this purpose, but in vivo data has not appeared (16).

It is generally assumed that increasing the amount of cholesterol in HDL is a worthwhile therapeutic goal since HDL-C has emerged as such a strong positive predictor of CHD. However, it is also possible, given the marked heterogeneity of HDL particles, that other components of HDL cause it to be "protective" and therefore could serve as a marker of CHD risk. One such component could be the amount of paraoxonase (PON) present on the HDL. This is an esterase that may be "anti-atherosclerotic" by virtue of its ability to degrade reactive species (e.g., lipid peroxides) generated by the oxidation of LDL. Recent PON knockout data in mice supports such a role (17). Therefore, agents which activate the transcription of PON or otherwise enhance its activity might be useful in the treatment of atherosclerosis.

Lipoprotein (a) (Lp(a)) - Lp(a) is a complex of the glycoprotein, apolipoprotein (a) and LDL. Apo(a) is covalently bound to the apoB-100 of LDL, and has close structural homology to plasminogen (18). Given the strong association between elevated Lp(a) levels and atherosclerotic disease (19), many investigators have been searching for an effective means to lower Lp(a) levels (20). However, it must be noted that there have been no clinical trials (primary or secondary) performed to determine the efficacy of Lp(a) lowering. To perform these trials and definitively determine the beneficial effects (if any) of Lp(a) lowering, an effective pharmacological agent must be found. The search for such agents has focused on agents that inhibit the synthesis of Lp(a) either by inhibiting the synthesis of apo(a) or by preventing the post transcriptional coupling of apo(a) to apoB-100. A number of compounds have recently been shown to affect apo(a) gene transcription. It has been shown that 9-cis retinal decreased Lp(a) levels in primary cynomolgus monkey hepatocyte cultures with an IC_{50} of 0.2 μM. It was also shown that apo(a) mRNA levels decreased (21). It was recently demonstrated that the apo(a) promoter contains a retinoid response element (RRE) and it is likely that this RRE mediates the effect that retinoids have on apo(a) mRNA expression (22). In a similar manner, raloxifene has also been shown to lower both plasma apo(a) levels in transgenic mice, expressing human apo(a), and the apo(a) mRNA levels in the livers of the same mice(23). Studies have shown that the assembly of Lp(a) occurs extracellularly at the cell surface of the hepatocyte (24). It has been postulated that this is a two step process, involving the formation of a loose complex between the lysine binding kringles of apo(a) and lysine residues in apoB-100, followed by formation of a disulfide bridge between Cys4057 of apo(a) and Cys3734 of apoB-100 (25). Non-specific agents, such as disulfide reducing agents, have been shown to interfere with the second step of this process and will not be discussed here (26). Amino acids, such as lysine and proline, and ε-aminohexoic acid (ε-AHA) have been shown to inhibit the formation of this loose complex (27). It has been shown that an analog of ε-AHA, tranexamic acid, lowers Lp(a) levels by 18.5% in a small eight patient study (28). Molecular modeling studies have characterized a 14 amino acid fragment of apo B-100 (P^{3732} SCKLDFREIQIYK) that provides the largest number of contacts with a molecular

model of the type-9 kringle in apo(a)(29). This peptide has formed the basis of an SAR study designed to identify additional peptides that can prevent the coupling of apo(a) to apoB-100. One of these peptides, PSIKLDFREIQCYK, inhibited the coupling of a recombinant apo(a) to LDL with an IC_{50} of 6 μM (30). The ACAT inhibitor, avasimibe (**8**), lowered Lp(a) levels, in cynomolgus monkeys, to 68% of control levels at a dose of 30 mg/kg after three weeks of dosing (31). The carboxyalkylether (**9**) lowered Lp(a) levels, in cynomolgus monkeys, by 78% after three weeks of dosing (32).



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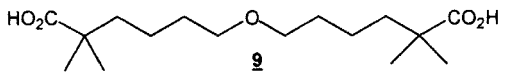

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COMBINATION THERAPIES

Given the widespread utilization of HMGRI's for the treatment of hyperlipidemia, it has become apparent that new strategies aimed at altering either plasma lipoproteins or directly affecting vascular disease progression would be used in combination with HMGRI's. The various combinations of HMGRI's with other agents such as bile acid sequestrants and niacin have achieved greater LDL lowering with little effect on other lipoproteins. Bile acid sequestrants in combination with fibrates or niacin are also useful but poor tolerance and side-effect profiles have limited their usefulness.

In preclinical studies, HMGRI's have been shown to limit the progression of atherosclerosis (33). This antiatherosclerotic activity appears to be related to their hypolipidemic activity; however, there may be ancillary properties of the compounds that can have a direct influence on lesion development. Most notably, they have been shown to limit smooth muscle cell (SMC) migration and proliferation (34-36) and this may be one mechanism for their direct antiatherosclerotic properties. ACAT inhibitors have also been shown to possess this property but unlike HMGRI's, which limit overall lesion size and extent, ACAT inhibitors specifically decrease lesion monocyte-macrophage enrichment (37). It has been shown that a co-administration of atorvastatin and the ACAT inhibitor, CI-976, acts synergistically to lower plasma cholesterol levels and to markedly reduce the extent, size and cellular composition of developing atherosclerotic lesions (38). In models of pre-established atherosclerosis, a combination of avasimibe (**8**) and simvastatin has been shown to not only directly blunt lesion progression but also promote regression of pre-established atherosclerotic lesions (39). Therefore, while HMGRI's are potent lipid-lowering agents capable of limiting the progression of atherosclerosis, it is also apparent that combination with other agents could serve to have a more profound effect on the underlying vascular pathology.

DIRECT ARTERY WALL INTERVENTIONS

Historically, direct artery wall interventions would be broadly defined as strategies aimed at limiting the progression or promoting regression of atherosclerosis irrespective of changes in plasma cholesterol levels. Conceptually, limiting the amount of atherosclerosis would appear beneficial; however, a new paradigm is emerging which focuses on altering the cellular and chemical composition of the developing lesion that improves its physical properties. For instance, strategies aimed at stabilizing

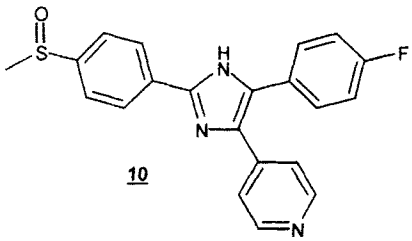
a vulnerable plaque by either limiting accumulation of macrophages or macrophage products such as matrix degrading enzymes should create a structurally more stable lesion. Several active cellular processes are occurring during the development of atherosclerosis, such as monocyte-endothelial cell adhesion events, SMC migration, proliferation and matrix production and macrophage foam cell formation all of which are potential sites of pharmacological intervention.

Atorvastatin in preclinical models reduced the extent, size and character of atherosclerotic lesions irrespective of changes in plasma cholesterol (33,40). Structural changes in atherosclerotic lesions following atorvastatin treatment have been attributed to alterations in SMC proliferation, migration, apoptosis as well as decreases in monocyte adhesion, proliferation and neointimal monocyte-macrophage accumulation (35, 41-45). Associated with reductions in monocyte-macrophage content are decreases in MCP-1 protein, and diminished AngII and TNF-α induced NF-κB activation and MCP-1 gene expression (45,46). Atorvastatin also downregulates vascular SMC expression of IP-10, a potent T-cell and monocyte chemoattractant which promotes T-cell adhesion to endothelial cells as well as monocyte-macrophage and SMC expression of matrix metalloproteinase (MMP –9 and MMP-1), respectively (47,48). Functional changes in vascular reactivity and protection of LDL against oxidation have also been noted (49,50). In addition, Atorvastatin ablated the hypercholesterolemia-induced reduction in vascular relaxation response to acetylcholine and it reduced prepro-ET-1 mRNA expression, ET-1 levels and overcame the oxidized-LDL-induced repression of nitric oxide synthase mRNA levels which resulted in elevated nitrite levels, an index of nitric oxide synthase activity (51, 52).

This brief review of the pleiotropic properties of atorvastatin highlights several potential areas of intervention that, if targeted directly, may further limit atherosclerosis progression and potentially cause lesion regression. The specific interventions can be categorized into 3 broad groups. Firstly, agents that alter lesion structure by preventing monocyte-endothelial cell interactions, smooth muscle cell proliferation and migration and monocyte-macrophage differentiation. Secondly, agents that inhibit the production or activity of molecules secreted by resident cells such as MMPs, IP-10 and MCP-1 and finally, agents that improve vascular reactivity by increasing nitric oxide synthase activity.

Adhesion of monocytes to endothelial cells is an initial event responsible for the accumulation of monocyte-macrophage foam cells within developing atherosclerotic lesions. Vascular cell adhesion molecule-1 (VCAM-1) has been implicated in atherosclerotic lesion development and associated with the lesion progression (53). The VCAM-1 gene contains binding sites for NF-κB and inhibitors of activation have been shown to effectively reduce VCAM-1 expression and in some cases monocyte adhesion (54). The anthraquinone, emodin, through inhibition of I-κB degradation decreased the TNF-induced monocyte-adhesion and endothelial expression of VCAM-1 (55). Anti-inflammatory agents such as curcumin blocked the adhesion of monocytes and expression of VCAM-1 when administered prior to TNF stimulation; however, attenuation was noted even when administered 1 hr after TNF(56). An inhibitor of NF-κB activation, pyrrolidine dithiocarbamate, was shown to limit VCAM-1 gene expression in cultured neonatal rat cardiac myocytes (57). Alpha-tocopheryl succinate, by suppressing NF-κB mobilization, has also been noted to inhibit cytokine-stimulated expression of VCAM-1(58). Protein tyrosine phosphatase inhibitors, such as, phenylarsine oxide, pervanadate, and diamide blocked TNF-dependent NF-κB activation and induction of VCAM-1 (59). Protein tyrosine kinase inhibitors, genistein and herbimycin A, inhibited endothelial cell expression of VCAM-1 and U937 cell adhesion (60). An inhibitor of p38 mitogen activated kinase, SB203580 (**10**), was shown to inhibit surface expression of VCAM-1 but to have no effect on mRNA levels suggesting that p38 MAP kinase pathways may be involved in the post-transcriptional

regulation of VCAM-1 (61). Combined inhibition of PDE3 and PDE4 by rolipram and ORG9935 (11), respectively, synergistically reduced VCAM-1 expression but either alone was ineffective (62). General antioxidants, such as resveratrol have also been found to inhibit VCAM-1 expression (63). While it is unclear whether inhibition of VCAM-1 expression through a variety of mechanisms would limit monoctye-endothelial cell adhesion in vivo, these data highlight that inhibitors of nuclear transcription factors and specifically, NF-κB, could provide novel therapeutics.



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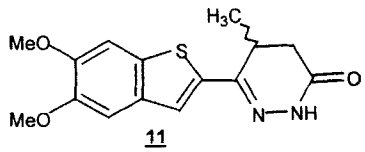

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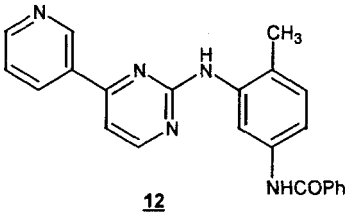
Another mechanism by which one can reduce monocyte-macrophage accumulation may relate to the inhibition of chemotactic signals. Monocyte chemotactic protein-1 (MCP-1) expression was reduced upon atorvastatin treatment and inhibitors of MCP-1 expression have been identified. Cilostazol, a cAMP phosphodiesterase inhibitor, has been shown to increase intracellular cAMP levels and reduce both basal and TNF-α-induced MCP-1 expression by human umbilical vein endothelial cells (64). Curcumin, a compound with both anti-inflammatory and anti-oxidant properties, was found to reduce both MCP-1 and IP-10 expression (65). Progesterone, losartan and irbesartan, and quercetin have all been shown to reduce the expression of MCP-1 by various cells (66-68). Thus, inhibitors of MCP-1 may be of utility for the treatment of atherosclerosis.

Since SMC's are a key component of the atherosclerotic lesion, inhibitors of SMC proliferation and migration may prove useful for the treatment of the disease. A potential mechanism for the observed effect of HMGRI's on atherosclerosis progression may relate to the reduction of isoprenoids involved in cell proliferation. Inhibition of tyrosine kinase with tyrphostins, has been shown to inhibit intimal hyperplasia in vein grafts (69). Epigallocatechin, by reducing the JNK-stress-activated protein kinase signal transduction pathway was noted to inhibit SMC proliferation in vitro (70). While prevention of SMC proliferation may be important in preventing restenosis and limiting SMC growth in the developing atherosclerotic lesion, inhibition of SMC migration may also be equally important. Tranilast, an agent shown to inhibit TGF-β secretion and antagonize the effect of TGF-β and PDGF on cell migration, has been shown to reduce the incidence of restenosis following angioplasty (71). Specific inhibition of PDGF receptor tyrosine kinase with CGP53716 (12) was found to inhibit tyrosine phosphorylation of PDGF receptor-α and –β in vitro and to limit SMC migration but not proliferation in the balloon-injured rat carotid (72). Therefore, one can conclude that inhibition of both SMC proliferation and migration might limit atherosclerotic lesion progression by not only directly reducing the number of cells but also by indirectly decreasing their secreted products such as matrix. However, efforts to titrate the level of inhibition may be necessary since complete ablation of SMC accumulation may predispose the lesion to plaque rupture due to a reduction in the size of the fibrous cap.

Efforts to limit and stabilize atherosclerotic lesions through inhibition of matrix remodeling are attractive and potentially useful in preventing plaque rupture. Matrix metalloproteinases (MMPs) have been localized to atherosclerotic lesions and they have been circumstantially implicated in promoting plaque destabilization due to their expression in macrophage-rich, rupture-prone regions of the plaque (73). The poorly bioavailable, peptide hydroxamic acid, GM6001(13), was noted to markedly reduce neointimal formation following balloon catheter injury (74).

Another area of potential investigation relates to the improvement of vascular reactivity. Nitric oxide synthase (NOS) induction in the arterial wall may promote vasodilation and could be associated in part with the beneficial outcome noted with the various HMGRI's. The JAK/STAT signal transduction pathway has been implicated as a positive effector of inducible-NOS (iNOS) in macrophages (75). Stimulation of iNOS and nitric oxide (NO) production by macrophages has been accomplished using liposomes containing the synthetic lipopeptides, JBT3002 or CGP31362 (76).



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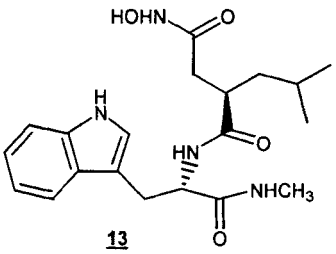

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CHALLENGES FOR THE FUTURE

Gene Therapy - There is an obvious genetic component in the development of atherosclerosis and the search, among the 1.4 million possible genes of the human genome, for the genes involved in the inheritance of atherosclerosis continues. To date, approximately 17 proteins and their corresponding 'candidate genes' have been discovered and implicated as being involved in lipoprotein metabolism and the development of atherosclerosis. These proteins/genes can be divided into three groups: apoproteins: lipoprotein processing proteins: and lipoprotein receptors (77). Various inherited disorders of lipoprotein metabolism have been identified and genetic mutations within the candidate genes have been correlated with abnormal lipoprotein subtypes (78). The goal of gene therapy is to correct this genetic mutation by the introduction of a correct gene that would express the normal protein and compensate for the defect in the endogenous gene defect. The greatest obstacle facing gene therapy is the effective delivery of the gene (79). Two methods are employed: in vivo delivery and ex-vivo delivery. This latter method has been utilized with some measure of success, in delivering the LDL receptor gene to a patient suffering from homozygous familial hypercholesterolemia (80). Many of the lipid disorders associated with the candidate genes (LDL-r, VLDL-r, LCAT, LPL, apo A1 and E) have been studied in animal models using the ex vivo approach (81).

For vascular targets, the direct delivery of a gene, in a suitable vector, to the target cell is emerging as the preferred method of gene delivery for targets at the artery wall. Early stage clinical trials using the VEGF (vascular endothelial growth factor) gene to stimulate angiogenesis in patients suffering from peripheral vascular disease shows early promise (82).

Surrogate Markers - With an increased understanding of vascular biology has come the need to identify surrogates of activity for direct-acting, anti-atherosclerotic agents. A surrogate may not be a direct measure of clinical benefit, but may be a substitute for and predict such benefit. It may therefore give information on the efficacy of a compound during therapy, without the need to determine the actual extent of disease, in this case, atherosclerosis. For example, if a compound down-regulates VCAM expression in endothelial cells, would it decrease the level of soluble VCAM (sVCAM) in plasma? In some studies, sVCAM concentrations correlate with the extent of atherosclerosis (83). Do drugs that directly inhibit macrophage ACAT in lesions inhibit ACAT in circulating monocytes? Would myeloperoxidase inhibitors inhibit lesion formation and reduce the amount of 3-chlorotyrosine in LDL (84)? It is anticipated that

new technologies might be used to identify novel surrogates of the extent of atherosclerosis (genomics, proteomics, etc) that could be monitored in the future.

Conclusions – In summary, the pleiotropic effects of statins have highlighted several new avenues of potential pharmacologic interventions. Compounds, while not all developed for the treatment of cardiovascular disease, have been highlighted above and further investigation is warranted to determine if they would be effective for the treatment of atherosclerosis. The challenge for the future development of direct acting antiatherosclerotic agents is the coincident development of surrogates or biomarkers of activity and/or non-invasive or semi-invasive image analysis modalities. While there are numerous imaging modalities such as intravascular ultrasound (IVUS), positron emission tomography (PET), magnetic resonance imaging (MRI), computed tomography (CT) and various spectroscopic and radiographic methods, clinical and preclinical utilization of such modalities for assessing atherosclerosis are in their infancy.

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Chapter 11. Current and Potential New Therapies for Chronic Obstructive Pulmonary Disease

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Introduction – Chronic obstructive pulmonary disease (COPD) is an umbrella term used to describe conditions of fixed airways disease. Study of COPD has lagged significantly behind that of asthma, although, in the past few years, driven by the unmet medical need and increased understanding of its pathogenesis, there has been a noticeable increase in research on COPD, including potential new drugs.

Definition, Epidemiology, Pathophysiology and Unmet Medical Need – COPD is a heterogenous rather than a single disease entity. It is defined officially by the American Thoracic Society as a condition "characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema; the airflow obstruction is generally progressive, may be accompanied by airways hyperreactivity, and may be partially reversible" (1); the generally irreversible nature of COPD contrasts with asthma, which is largely a reversible disease. Chronic bronchitis is defined clinically as the presence of chronic productive cough for 3 months in each of two successive years (2), whereas emphysema is defined anatomically as abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls without obvious fibrosis (3).

COPD is a very common, serious condition with significant morbidity and mortality. In the US it affects about 15 million people (approximately 12.6 million possess chronic bronchitis and about 2 million have emphysema) and in 1991 it was the 4th leading cause of death (>85,000). From 1979 to 1991 the death-rate in the US increased by 33% (4). The major risk factor for COPD is cigarette smoking; it has been estimated to account for 80-90% of the risk for developing COPD (1,5). However, only about 15% of smokers get COPD. Other risk factors include passive smoking, pollution, occupational factors, sex, race and socioeconomic status (1). There is only a single genetic defect which results in COPD: alpha$_1$-antitrypsin deficiency which is responsible for <1% of COPD prevalence (1).

The current understanding of the mechanisms underpinning COPD is rather limited. However, several types of immune and inflammatory cells, in particular neutrophils, macrophages and T-cells, have been proposed to contribute to the pathophysiology of this disease. COPD is predominantly a small airways disease whose features include mucous gland hypertrophy and hypersecretion, inflammation (mononuclear-induced), fibrosis, goblet cell metaplasia and smooth muscle hypertrophy (in terminal bronchioles) (1). The impact and manifestations of these characteristics are mucus secretion, cough, bronchoconstriction and lung remodeling. In addition, pulmonary vascular disease, notably pulmonary hypertension, is a common, potentially serious complication of COPD (6,7).

CURRENT THERAPIES

Present therapies for COPD are varied, but not very effective, and they are similar to those for asthma. The range of treatments include β-adrenoceptor agonists, anticholinergics, theophylline, corticosteroids, cromolyn and nedocromil, mucokinetic or mucolytic agents, and long-term oxygen therapy.

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β-Adrenoceptor Agonists – The β-adrenoceptor agonists elicit some bronchodilation in COPD, although it is less than that obtained in asthma. Furthermore, this class of compounds has no significant impact on the progression of COPD (1).

Anticholinergics – Topical anticholinergics, typified by ipratropium, produce bronchodilation in COPD, providing evidence of enhanced parasympathetic drive. Although ipratropium may be a more effective bronchodilator than β-adrenoceptor agonists, it also has no influence on the progression and decline in the disease (1).

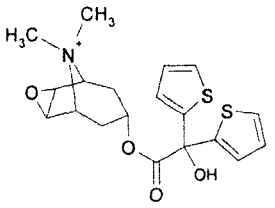
Theophylline – Oral, sustained release theophylline is widely used in COPD. In addition to a bronchodilator effect, there is evidence of anti-inflammatory actions with theophylline (1). Furthermore, by virtue of its influence in the cardiovascular system, theophylline may be of particular utility in COPD patients with cardiovascular complications (8). However, COPD progression is not altered by theophylline.

Corticosteroids – Corticosteroids, are the mainstay of non-bronchodilator therapy for asthma. However, their utility in COPD is less clear; there is evidence from several reports that oral corticosteroids are effective in a minority (20-30%) of patients (8). There is conflicting information on the ability of corticosteroids to modulate the progression of COPD (9). Three chronic, multi-center studies with aerosol corticosteroids in COPD have recently been conducted. Assessment of the data presented to date demonstrated no significant effects on the decline of FEV_1 (10).

POTENTIAL NEW THERAPIES

Novel Combinations of Current Therapies – It has been common practice to utilize treatments in combination for COPD. For example, there is evidence of increased efficacy of combinations of the bronchodilators, β-adrenoceptor agonists, cholinergic receptor antagonists and theophylline (11). Such a combined therapy has recently been introduced: Combivent, which is an aerosol formulation of the β-adrenoceptor agonist, salbutamol, and ipratropium (12).

Selective Muscarinic Receptor Antagonists – Based on the importance of muscarinic receptors in regulating airway tone and secretions (13), and the demonstrated clinical utility of ipratropium in COPD, there is significant interest in identifying and developing either longer acting, more potent non-selective muscarinic antagonists than ipratropium, such as tiotropium (1) or oxitropium (2), or more subtype-selective muscarinic antagonists, such as revatropate (3) or darifenacin (14-16).



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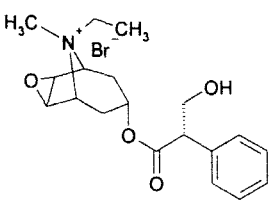

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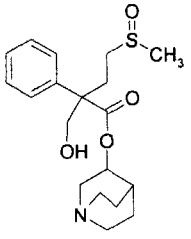

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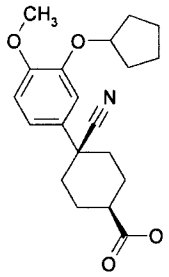
The lung expresses at least three subtypes of muscarinic receptors. M1 receptors are located primarily in the parasympathetic ganglia where their activation facilitates transmission through the ganglia, M2 receptors are found on presynaptic cholinergic nerve terminals, and function to inhibit acetylcholine release, and M3 receptors are present on the smooth muscle and elicit contraction (13). It has been speculated that selective blockade of M1 or M3 receptors should decrease the

cholinergic tone of the lung while antagonism of M2 receptors might enhance it. Revatropate (3) is a M1/M3 receptor-selective muscarinic antagonist with about 50-fold lower potency against M2 receptors (16). In animal models, revatropate (3) inhibited acetylcholine-induced bronchoconstriction but, unlike ipratropium, did not potentiate vagally-induced bronchoconstriction (16). Furthermore, in initial clinical trials for COPD, inhaled revatropate (3) produced a similar increase in FEV_1 as ipratropium (17). Tiotropium produced a significant, long-lasting bronchodilation in patients with COPD resulting in clinical effectiveness with once-daily dosing (14).

Phosphodiesterase4 (PDE4) Inhibitors – PDE4 belongs to the family of proteins, phosphodiesterases, which are responsible for the metabolism and inactivation of the intracellular second messengers, cyclic AMP and cyclic GMP; PDE4 catalyzes specifically the breakdown of cAMP. PDE4 is the predominant isozyme found in immune and inflammatory cells in the lung and contributes significantly to the metabolism of cyclic AMP in airway smooth muscle (18). Extensive preclinical research has provided strong rationale to suggest that selective PDE4 inhibitors may offer an effective novel approach for the treatment of COPD. This class of compounds combines bronchodilatory and anti-inflammatory activity, concomitant with modulation of the neuronal control of the lung (18). Unfortunately, the clinical utility of the first generation PDE4 inhibitors, such as rolipram, was limited by their propensity to cause gastrointestinal side effects (19, 20). The challenge to drug discovery has been the identification of second generation inhibitors that maintain the therapeutic actions of the earlier compounds but with a reduced potential to produce the characteristic side effects. One strategy to obtain this desired profile has been to design compounds with a reduced affinity for one form or conformation of PDE4, referred to as HPDE4, while maintaining or increasing the activity for another form of the isozyme, known as LPDE4 (19, 21). Inhibition of HPDE4 has been associated with production of the side effects of PDE4 inhibitors whereas inhibition of LPDE4 correlates with the beneficial actions of these compounds (19-22). Utilization of this approach has identified several molecules that, in preclinical models and in selected cases, clinical studies, maintained the bronchodilatory activity and anti-inflammatory activity of rolipram with a marked reduction in the production of gastrointestinal side effects; these compounds include SB 207499 (Ariflo^{TM}) (4), CDP840, V11294A (5), CP-220,629 (6), roflumilast (18, 20, 23-25).



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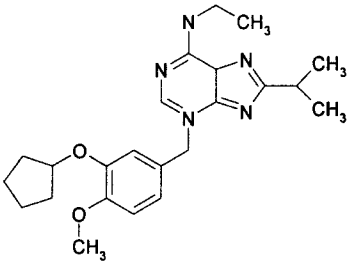

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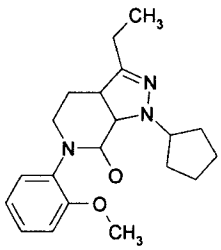

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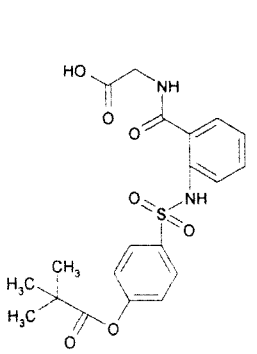
For example, in preclinical studies, SB 207499 (4) and CP-220,629 (5), demonstrated anti-inflammatory activity at doses that did not produce emesis (23,26). In initial clinical trials, oral administration of SB 207499 (4), at doses that were well tolerated, resulted in a marked improvement in both quality of life measures and lung function in individuals who were diagnosed with poorly reversible COPD (27, 28).

Proteinase Inhibitors – Proteinases can be classified into four broad categories: serine, cysteine, aspartic and matrix metalloproteinases. Serine proteinases,

especially neutrophil elastase, are believed to play a major role in the destruction of lung parenchyma observed in emphysema and on the basis of their mucus secretagogue activity, may contribute to the mucus hypersecretion seen in chronic bronchitis (29-31). Most drug discovery efforts on proteinases have focused on the identification of potent and selective elastase inhibitors; examples of these compounds include ONO-5046 (7), ICI 200,355 (8) and MR899 (9) and TEI-8362 (32, 33). In animal models, administration of these inhibitors prevent elastase-mediated lung damage (33) and inhibit mucus hypersecretion (31). In the only clinical report, administration of MR899 (9) for 4 weeks to individuals with COPD did not modify the level of plasma elastin-derived peptides or urinary desmosine, two biochemical markers associated with lung destruction (34).



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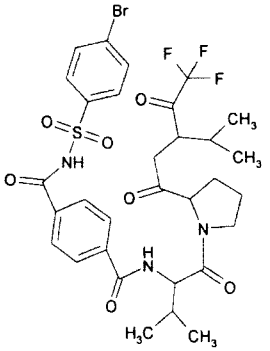

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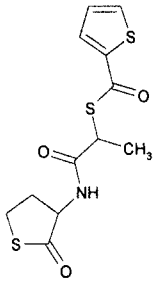

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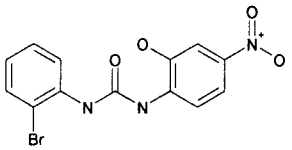
Other potentially important proteinases include cysteine proteinases in alveolar macrophages, such as cathepsin S and cathepsin L. For example, cathepsin S degrades elastin and has similar activity to that of neutrophil elastase (35). Furthermore, increased levels of cathespin L activity have been detected in the bronchoalveolar lavage fluid of smokers with subclinical emphysema (36). Other potential targets are the matrix metalloproteinases, which are released from activated neutrophils and macrophages. These are a 15-member family of secreted or membrane-bound enzymes, which can degrade most components of the extracellular matrix.

Chemokine Antagonists – The inflammatory cells implicated in COPD include neutrophils, $CD8^+$ T cells and macrophages (37-39). Thus, one approach for the treatment of this disease would be to target those molecules involved in the recruitment and/or activation of these inflammatory cells. The chemokines represent such a family of target proteins (40). They are approximately 8-12 kDa in size and are produced by a variety of cells in response to pro-inflammatory stimuli such as IL-1β and TNFα (41). There are approximately 30-40 distinct chemokines that are subdivided into 4 families known as CXC, CC, CX3C and C (40). These proteins produce their effects through activation of members of the G-protein coupled, seven transmembrane-spanning superfamily of receptors (40, 42).

The evidence supporting the functional role of chemokines in the pathogenesis of COPD is developing. For example, levels of IL-8, the prototypical CXC chemokine that recruits and activates neutrophils through an interaction with CXCR1 and/or CXCR2 (40), are elevated in bronchoalveolar lavage fluid (BAL) obtained from individuals with COPD (38). Administration of neutralizing antibodies to IL-8 reduces both neutrophil influx and damage in several animal models of acute lung injury (43). Since human neutrophils express both CXCR1 and CXCR2, it is important to determine which receptor, or receptors, are responsible for the effects

of IL-8 and related chemokines in the lung. Results obtained from CXCR2 knockout mice and those obtained with a non-peptide, selective CXCR2 antagonist, SB 225002 (**10**), provide evidence that CXCR2 is essential for the recruitment of neutrophils, whereas activation of CXCR1 increases elastase release and superoxide production (44-46). These initial observations support the potential utility of selective CXCR1 and/or CXCR2 antagonists in the therapy of COPD.



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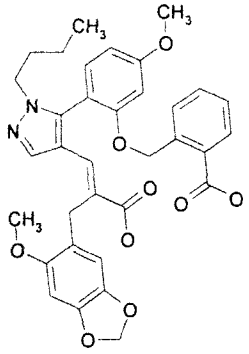
There is limited information on the importance of the CC chemokines and their receptors in COPD. Recently it was observed that the MIP-1β concentration was increased only in BAL fluid obtained from smokers with chronic bronchitis, whereas MCP-1 levels were elevated in BAL fluid from smokers regardless of the absence or presence of bronchitis (47). Several distinct structural classes of CCR receptor antagonists have been disclosed that are reportedly selective for CCR1, CCR2, CCR3 and CCR5 (42). It is not yet known if these compounds are active in any animal models of lung diseases.

Endothelin Modulators – Endothelin-1 (ET-1) is a 21-amino acid peptide isolated from cultured endothelial cells in 1988 (48). The diverse biological effects are ET-1 are mediated via two receptors, designated ET_A and ET_B, which belong to the superfamily of G-protein coupled, seven transmembrane–spanning receptors (49). There is accumulating evidence for a potential pathophysiological role of ET-1 in pulmonary diseases, notably asthma and pulmonary hypertension. Pulmonary vascular disease is a common, potentially serious complication of COPD; it has been estimated that >50% of COPD patients who are over 50 years old will develop pulmonary hypertension (7). The two features of pulmonary hypertension, namely pulmonary vasospasm and vascular remodeling, are mimicked by ET-1. Thus, ET-1 is a potent contractile agonist in human isolated blood vessels and increases proliferation in human pulmonary artery (50-52). The former influence is elicited primarily via activation of ET_A receptors, with evidence for a contribution from ET_B receptors , and the mitogenic effect of ET-1 is ET_A receptor-mediated (50,52,53). In addition to a possible role in the vascular component of COPD, other potentially pathophysiologically relevant effects of ET-1 *in vitro* include an ability to increase mucus secretion, recruit and activate neutrophils and macrophages, and potentiate nerve-induced responses (54-56). Several studies have demonstrated an increase in ET levels or expression in children or adults with pulmonary hypertension, or after exposure of animals or humans to hypoxia (54, 55)

Two approaches have been employed in the search for ET-1-directed therapeutics: (i) inhibitors of endothelin-converting enzyme (ECE), which mediates the synthesis of ET-1 from its precursor, big ET-1 (49, 54, 55); (ii) receptor antagonists of the effects of ET-1 at the end-organ level. The latter strategy has been the more successful one. Thus, several potent and selective nonpeptide receptor antagonists from different structural classes have been identified (57-63). Examples of these, ET_A receptor-selective or ET_B receptor-selective compounds, or mixed ET_A/ET_B receptor antagonists (**11**-**16**). Many have undergone extensive pre-clinical assessment, including examination in animal models of pulmonary hypertension. For example, BQ-123, SB 217242 (**15**) and bosentan (**14**) reverse and/or prevent the increase in pulmonary artery pressure and vascular remodeling

elicited by acute or chronic hypoxia or by monocrotaline in rats or guinea pigs (60-62). A key issue is what is the desired optimal profile, with respect to ET receptor subtype-selectivity, of a compound for COPD: ET_A receptor-selective, ET_B receptor-selective or combined ET_A/ET_B receptor-selective antagonist?

A. ET_A Receptor-selective



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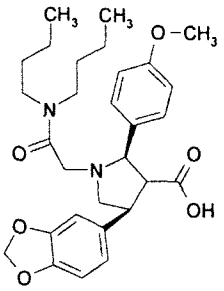

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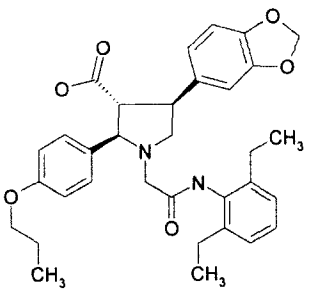
B. ET_B Receptor-selective



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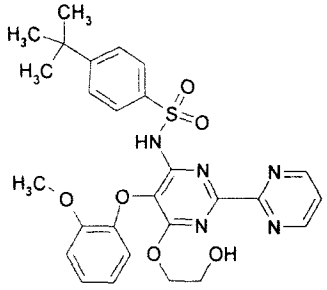
C. Mixed ET_A / ET_B Receptor Antagonists



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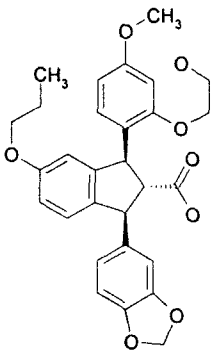

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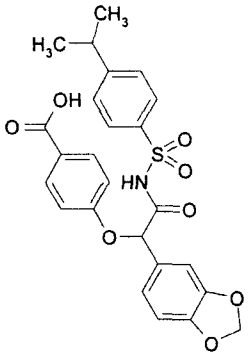

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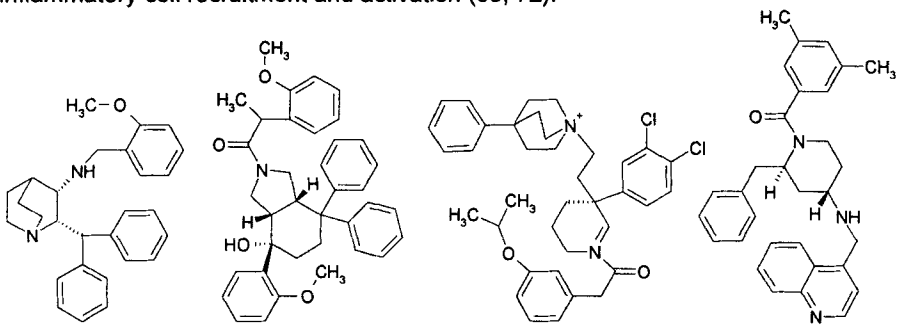
Tachykinin Receptor Antagonists – The tachykinins are a family of small peptides – the main members being substance P, neurokinin A (NKA) and neurokinin B (NKB) – found in the central and peripheral nervous systems. Predominant locations are the peripheral endings of capsaicin-sensitive primary afferent neurons (unmyelinated C fibers), which innervate many sites in the periphery, including the lung (67). The diverse effects of the tachykinins are elicited by activation of three known receptors, NK-1, NK-2 and NK-3, which belong to the superfamily of G-protein coupled, seven transmembrane–spanning receptors (69). Activities of the tachykinins in the lung include neurogenic inflammation, plasma extravasation, mucus secretion, bronchoconstriction, inflammatory cell chemotaxis and activation (69). In addition, substance P levels were elevated in sputum samples from patients with COPD (70).

The first nonpeptide tachykinin receptor antagonist reported was CP-96,345 (17), an NK-1 receptor antagonist, identified via high-throughput screening (71). Since the discovery of this pioneer compound, several potent and selective nonpeptide NK-1 receptor antagonists from different structural classes have been

identified (72). The effects of the tachykinins induced by activation of NK-1 receptors in the lung include mucus secretion, microvascular permeability and inflammatory cell recruitment and activation (69, 72).



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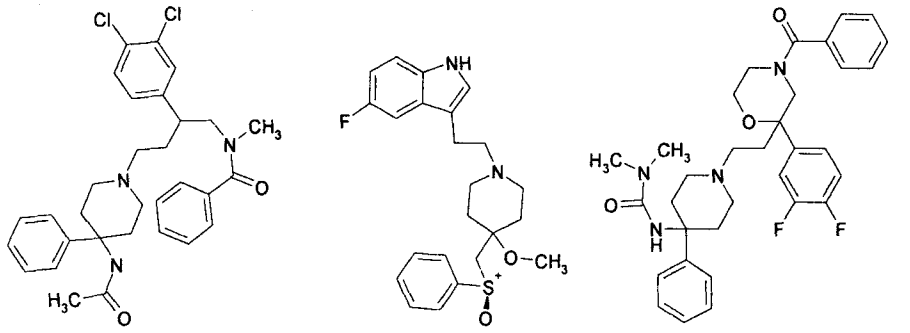
Not long after the identification of CP-96,345 (17), Sanofi presented information on a potent and selective, nonpeptide NK-2 receptor antagonist, SR 48968 (21) (73). Subsequent potent members of this class of compounds include GR 159897 (22) and SR 144190 (23) (72, 74, 75).



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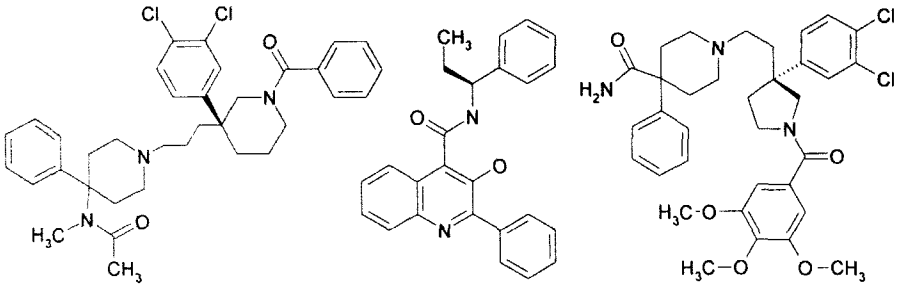
The most prominent effect of NK-2 receptor activation in the lung is bronchospasm; tachykinin-induced contraction in human isolated airways is mediated exclusively via NK-2 receptors (69, 72). In addition, NK-2 receptors may be involved in activation of alveolar macrophages, neurogenic inflammation and potentiation of nerve-induced responses (69, 72). NK-2 receptor antagonists, such as SR 48968, are effective in animal models of airways hyperresponsiveness and cough (69, 72, 76, 77).

A few potent and selective NK-3 receptor antagonists have been identified, exemplified by SR 142801 (24) (78) and SB 223412 (25) (79). The role of NK-3 receptors in the lung has not been extensively researched. Nevertheless, NK-3R mRNA has been demonstrated in human lung (80), and electrophysiological analysis indicates the presence of NK-3 receptors in guinea-pig bronchial parasympathetic ganglia with evidence for a modulatory role on neuronal inputs (81). SR 142801 is effective in guinea pig models of cough and bronchial hyperreactivity (82, 83).

In view of the diverse effects of activation of the individual tachykinin receptors in the lung, several of which may be pertinent to a role in COPD, a compound which interacts with more than one receptor may be more effective clinically than one which is selective for only one of the tachykinin receptors. An example of a combined nonpeptide NK-1/NK-2 receptor antagonist is MDL 105,212A (26) (84).



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Conclusion – COPD remains a major medical challenge with the need for novel therapeutics to control the different components and pathophysiological features of this prevalent and serious disease. Currently, several research approaches are being investigated directed towards modulation of the cells, mediators and processes which are thought to be responsible for the mucus secretion, cough, bronchospasm and lung remodeling that are characteristic of COPD. Recently introduced new drugs have been combinations (e.g., Combivent) and improved versions of existing therapies. Most potential new modalities currently undergoing investigation have only been evaluated preclinically. However, the positive clinical data with the PDE4 inhibitor, Ariflo™, suggests the real potential of the introduction of a member of a completely new class of compound for COPD in the near future.

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SECTION III. CANCER AND INFECTIOUS DISEASES

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Chapter 12. The Urokinase-type Plasminogen Activator and Its Receptor in Cancer

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Introduction - Proteolytic enzymes and their inhibitors play a significant role in invasive diseases such as cancer growth and metastasis as well as degradative diseases such as arthritis (1,2). In addition, these proteins have been implicated in the process of inappropriate neovascularization or angiogenesis, suggesting a role in tumor growth and ocular diseases, such as diabetic retinopathy and macular degeneration (3). Two proteolytic systems have been studied most extensively in these areas: the matrix metalloproteinases (MMPs), and the urokinase-type plasminogen activator (uPA) system. The first of these has been recently reviewed in Ann. Rep. Med. Chem. (4). Since this is the first review in this series which focuses on the uPA system in cancer invasion and metastasis, a somewhat more extensive consideration of historical data will be presented along with the state of the art in the development of enzyme inhibitors and receptor antagonists.

MOLECULAR COMPONENTS OF THE UROKINASE SYSTEM

The process of plasminogen activation is central to both extracellular matrix remodelling and cell invasiveness, as well as fibrinolysis. The former processes are largely mediated via the urokinase-type plasminogen activator (uPA), whereas the tissue-type enzyme (tPA) is largely responsible for fibrinolysis in the vascular space. Both of these serine proteases cleave the inactive pro-enzyme plasminogen uniquely at the Arg^{570}-Ser^{571} bond to generate the wide spectrum serine protease, plasmin, which is capable of degrading most components of the extracellular matrix, either directly, or indirectly. In the latter case, recent studies in plasminogen deficient mice have suggested that plasmin is the primary *in vivo* activator of a number of matrix metalloproteinases, specifically MMPs 3, 9, 12, and 13 (5). Although plasminogen activation can occur with just the activator and substrate present, both of the activators are thought to act *in vivo*, primarily in association with either the cell surface (in the case of uPA) or fibrin (in the case of tPA). In both cases, plasminogen is also bound to the cell surface or fibrin via its lysine binding sites in a relatively low affinity but high capacity interaction (6). Cell surface bound plasmin is resistant to inactivation by its cognate inhibitor, the serpin α-2-antiplasmin (7).

Pro-urokinase is synthesized and secreted as a 500 amino acid largely inactive precursor, which can be activated by proteolysis at the K^{159}-I^{160} bond, generating the two-chain, disulfide bond-linked active enzyme. The protein consists of 3 domains, an N-terminal epidermal growth factor-like domain, a single kringle, homologous to those in plasminogen, and a serine protease domain, which is a member of the trypsin-like serine protease family. Structural studies have elucidated the 3-dimensional structure of the amino-terminal fragment of uPA (the first 2 domains, residues 6-135) by NMR methods (8) and the catalytic, serine protease domain structure has been determined by X-ray methods (9). Although no 3-D structure of the entire molecule has been determined, the domains of uPA have been shown to have considerable independent motion by NMR (10).

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The cell surface localization of uPA was first described in 1985 (11), and the identification of the receptor for uPA was determined by cDNA cloning of the protein in 1990 (12). This binding was shown to be reversible and of high affinity (Kd = 1 nM), to show significant species specificity (13), and to occur for both pro-uPA and the two-chain active form (11). The urokinase receptor (uPAR, CD87) is synthesized as a 313 amino acid precursor, which is modified at the amino terminus by removal of the signal peptide and at the carboxyl terminus by cleavage at approximately residue 283 and subsequent addition of a glycosyl-phosphotidyl inositol (GPI) anchor (14). The urokinase receptor is a member of the Ly6/CD59 family of GPI-anchored proteins, and consists of 3 highly disulfide linked domains of approximately 90 amino acids, each of which is homologous to CD59, for which an NMR structure has been solved (15). The disulfide bonding pattern for domain 1 of uPAR has been shown to be the same as for CD59, although only 4 of the 5 cysteine pairs are present in this domain of uPAR (16). No three dimensional structural information is available for uPAR, although several models have been proposed based primarily on the snake neurotoxins (17).

Active two-chain uPA is inactivated at the cell surface primarily by plasminogen activator inhibitor-1 (PAI-1), a prototypical member of the serpin class of serine protease inhibitors (18). PAI-1 is stabilized in its active form by binding to vitronectin, an extracellular matrix and serum component (19). UPA-PAI-1 complexes, bound to uPAR, are then cleared from the cell surface by members of the LDL-receptor related protein family, such as LRP (20). This process appears to be completed by the degradation of the protease-inhibitor complexes in lysosomes, and the re-cycling of the empty receptor back to the cell surface (21).

Recent results have shown that uPAR can associate with a number of other proteins, in addition to uPA (22), and that in a variety of cell types uPAR dependent signal transduction is observed, despite the absence of an intracellular domain (23). The best characterized interaction is that between uPAR and vitronectin (24). The uPAR-vitronectin interaction is also of high affinity and requires the N-terminal somatomedin B domain of vitronectin (25). Both uPA and vitronectin can be bound to uPAR simultaneously, and it appears that binding of uPA enhances the extent or affinity of vitronectin binding (26). To further complicate matters, uPAR and PAI-1 compete with each other for binding to vitronectin (27), and PAI-1 blocks the binding of the integrin $\alpha v\beta 3$ to the RGD sequence in vitronectin (28). The observation that high PAI-1 levels are a bad prognostic marker in a variety of tumors is perhaps linked to the ability of high PAI-1 levels to cause release of cells which are bound to vitronectin via uPAR (27,29). Additional studies have implicated uPAR in interactions with other proteins involved in cell: matrix interactions and cell migration, such as caveolin and especially β-1 and β-2 integrins such as Mac-1 (30-33).

THE UROKINASE SYSTEM IN CANCER

Given that a significant fraction of cancer patients present with occult metastatic disease, therapeutics focused on the inhibition of the metastatic process are likely to be of considerable benefit. Extracellular matrix remodelling is key to a number of steps in this process, and thus, inhibitors of MMPs such as Marimastat are in clinical development (34). Components of the uPA dependent plasminogen activation system, including uPA, PAI-1, and uPAR have all been shown to be upregulated in tumor versus normal tissue in a wide variety of human cancers, and increased levels of these molecules are independent prognostic indicators for survival and relapse in a number of major cancers, including those of the breast, colorectal, gastric, and urogenital tracts (reviewed in 35-37). An emerging paradigm appears to be that different components of this system are expressed in different cell types, depending on the specific cancer studied (reviewed in 35). Thus, a paracrine relationship exists between cells expressing uPA and uPAR in colorectal and breast cancer (38,39). The urokinase receptor was independently identified as the antigen for the Mo3 antibody, a marker of monocyte activation (40). The relevance of this observation is further supported by the high levels of uPAR seen on tumor-associated macrophages in breast carcinoma

samples (41). It has also been demonstrated that angiogenic growth factors, such as bFGF and vEGF, upregulate the components of the PA system in endothelial cells, especially uPA and uPAR (42), and it has recently been shown that hypoxia, one of the key physiological triggers of neovascularization, also upregulates uPAR expression (43). Thus, the uPA system is expressed in tumor cells, tumor-associated inflammatory cells, and endothelial cells, all of which represent potential targets for intervention in the process of tumor progression.

IN VIVO STUDIES

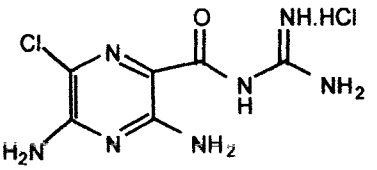
A number of different groups have shown that inhibition of urokinase enzyme activity or the interaction between uPA and uPAR can reduce tumor growth and/or metastasis in rodent models (reviewed in 35). These approaches have largely used biological reagents (anti-sense, ribozymes, antibodies, soluble receptor, inactive uPAR receptor ligands). In addition, a direct effect of uPAR antagonists on angiogenesis has been demonstrated *in vivo* by several groups (44-46). UPAR has also been shown to be critical for tumor cell intravasation in a chick chorioallantoic membrane assay (47). UPA-/- and uPAR-/- mice are viable and fertile, and show relatively mild fibrinolytic deficits (48,49). An additional deficit in neutrophil migration has been shown in uPAR-/- mice (50). The uPA-/- mice also show a reduction in the ability to clear some infectious agents (51). Most recently, plasminogen deficient mice have been shown to have reduced metastasis in a spontaneous mammary carcinoma model (52). These results together suggest that the uPA system represents an excellent target for interfering with the processes of tumor progression and metastasis (35).

Two molecular approaches can be envisioned for inhibition of uPA-dependent plasminogen activation: urokinase enzyme inhibitors and uPA receptor antagonists. It is premature to conclude which of these approaches may ultimately lead to approvable pharmaceutical agents, as neither approach has led to clinical candidates as yet, and the complex set of interactions in this system makes any predictions risky.

UROKINASE ENZYME INHIBITORS

Given the precedent for inhibition of other serine proteases in the fibrinolytic and clotting cascades, such as thrombin, the inhibition of the urokinase enzyme is an excellent target to block this cascade (53). The key issue is to obtain highly potent and selective inhibitors, especially with respect to other members of the trypsin-like family of serine proteases, such as tPA. The likelihood of success, in this regard, has been increased by the determination of the three dimensional structures of the protease domains of both uPA and tPA (9,54) . Recent work has shown that substrates which are highly selective for uPA vs tPA, and which are more reactive than the target sequence in plasminogen, can be obtained via bacteriophage display methods (55). A variety of approaches have been taken to generate urokinase enzyme inhibitors from exisiting protease inhibitor scaffolds. Thus, the bacterial protein ecotin has been modified using bacteriophage display methods to generate a highly potent uPA enzyme inhibitor (56). The selective substrates for uPA defined above have been used to generate a derivative of PAI-1 which is now significantly uPA selective (57). Other scaffolds such as the Kunitz type inhibitors have been used as well (58). In addition, fusions of protein uPA inhibitors with the amino-terminal fragment of uPA, creating molecules which bind to the uPA receptor and also inhibit uPA enzyme activity, have been described (59,60). One of these, a fusion of urinary trypsin inhibitor to ATF, has been shown to be active in *in vitro* invasion assays (61).

Small molecule inhibitors of uPA have been relatively poorly characterized, especially as compared to other serine proteases such as thrombin and leukocyte elastase. All of the inhibitors described to date have an amidino functionality, which likely makes contacts in the P1 pocket, in analogy to the arginine guanidino side chain. The first competitive inhibitor described was the diuretic, amiloride (1), which has an IC_{50} in the low μM range (62).



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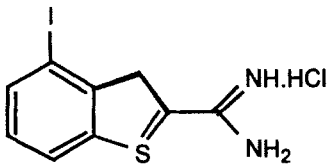

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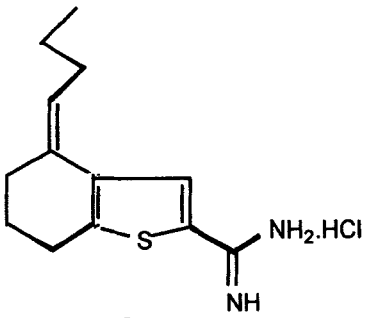
Somewhat related molecules were derived by Eisai, leading to the substituted benzothiophenes such as B-428 (2) (63) which show activity in the nM range, are more than 100 fold selective for uPA over tPA, and show activity in animal models alone and in combination with tamoxifen (64). More recently, Fujisawa has described related benzothiophene derivatives such as molecule 3, which has an IC50 of 0.47 uM (65):



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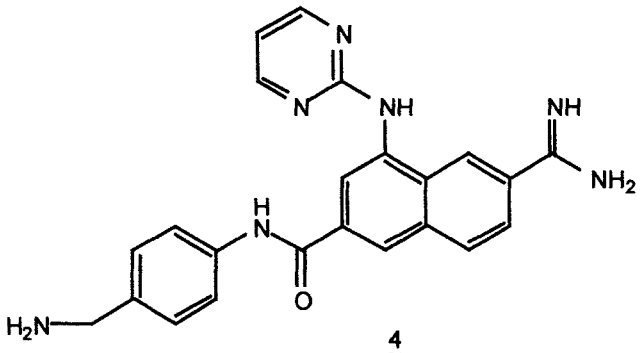
Finally, a new class of inhibitors of uPA using a naphthamidine scaffold have been described in the patent literature (66). These molecules, typified by 4, are highly potent, having Ki values in the sub-nM range.



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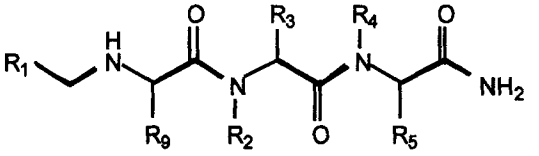
It should be noted that all of the uPA enzyme inhibitors will only react with active, 2-chain uPA, and will not affect any non-proteolytic functions of pro-uPA. It is also likely that receptor-bound inactive uPA will not be subjected to PAI-1:LRP mediated clearance and degradation.

UROKINASE RECEPTOR ANTAGONISTS

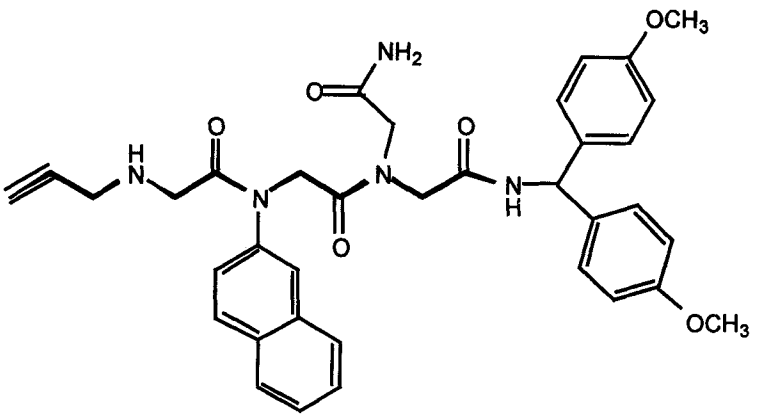
An alternative strategy to interfering with the uPA:uPAR pathway is to use molecules which compete for the binding of uPA to uPAR. Such molecules would reduce the rate of plasminogen activation by preventing uPA binding, and hence, the reciprocal cell surface activation of pro-uPA and plasminogen (7). A recent review of this approach has appeared (67). The domain organization of uPA originally suggested that

fragments of uPA would retain receptor binding without proteolytic activity, and would function as inhibitors of plasminogen activation. The interaction between uPA and the uPA receptor was first shown to involve the EGF-like N-terminal domain of uPA by deletion studies (68). The interaction was further localized to the central 2-stranded β sheet by peptide synthesis studies (69). In particular, peptides spanning the sequence from residues 12-32 showed significant binding in a competition assay format (IC_{50} = 100 nM). This region was later shown to be in an omega loop conformation by NMR studies of the amino-terminal fragment of uPA (8). It was also shown that the stringent species specificity of the uPA: uPA receptor interaction is also localized to this region (70). The analogous peptide from murine uPA was shown to have some activity in preventing metastasis in the Lewis lung carcinoma model (71). The synthesis of the EGF-like domain by recombinant yeast and its characterization, showed that the minimal binding domain for full binding affinity (Kd = 0.25 nM) is the first 48 residues of huPA (72). Interestingly, this molecule is not fucosylated at Thr-18 as is uPA from mammalian sources, a post-translational modification which has been implicated in uPA dependent mitogenesis in some human cell lines (73,74). A variety of fusion proteins have been prepared by a number of groups using either the EGF-like domain or the amino-terminal fragment of uPA to generate reagents which bind to the uPA receptor with high affinity and can be used in *in vivo* tumor studies (44,45,75).

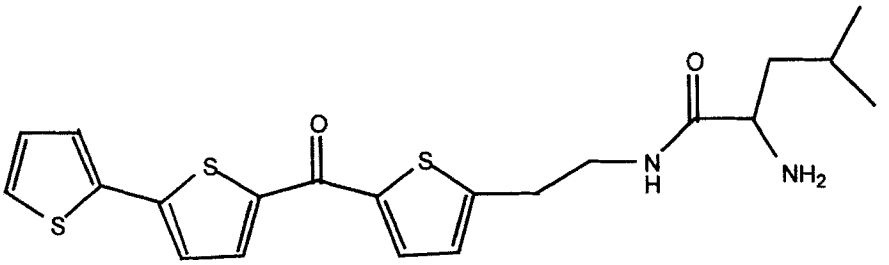
Molecules which are not derived from uPA but which inhibit the interaction between uPA and uPAR have been identified by a number of methods. Bacteriophage display libraries have been used to pan cells overexpressing uPAR and these studies led to the identification of novel 15-mer peptides which compete with uPA binding with IC_{50s} in the low nM range (76). Chemical and photo-activated cross-linking of derivatives of these peptides have identified residues in both domains 1 and 3 of uPAR as being involved in peptide binding (77). These studies are the first to elucidate why the intact uPAR is necessary for peptide, and by extension, uPA binding. The screening of combinatorial chemistry libraries of oligomeric substituted glycinamides has led to the identification of receptor antagonists in the nM range (78,79). These molecules have the generic structure:



The most potent compound, CHIR 11509, 5, (IC50 = 20 nM) has the following structure:



Very recently two additional patents have disclosed compounds which inhibit the uPA:uPAR interaction. The first class are thiophene derivatives, typified by **6**, with IC50s in the low uM range (80).



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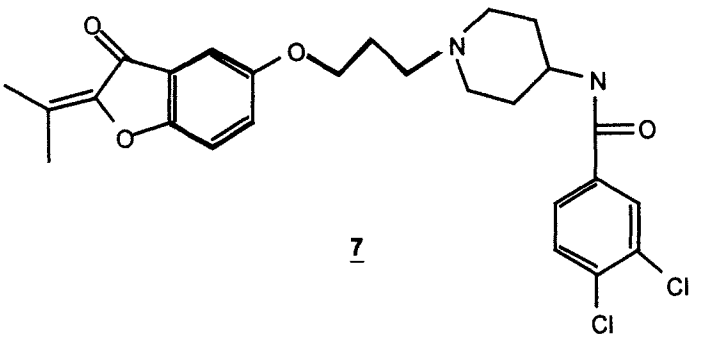
More interesting are the cumaranone derivatives, such as **7** which are claimed to have IC50s in the low nM range (81).



7

Other molecules, including porphyrins have been described in the patent literature (82) but no quantitative data on their potency has been disclosed.

Prospects and Conclusions - The role of the uPA:uPA receptor system in development, biology, and pathology has been significantly elucidated over the last decade. The position of this system, with the MMPs, as a key one in tumor metastasis and angiogenesis seems clear. The initial approaches to modulating the system have used protein or peptide reagents - although these molecules are less suitable for systemic, chronic dosing, they may have advantages in reduced toxicity and increased specificity. The development of orally available, small molecule inhibitors is still in the future. It seems likely that the first molecules which inhiibit uPA enzyme activity or block uPA binding to the uPA receptor will reach clinical trials in the first year of the new millenium.

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Chapter 13. Progress in the Search for Anti-HCV Therapeutics

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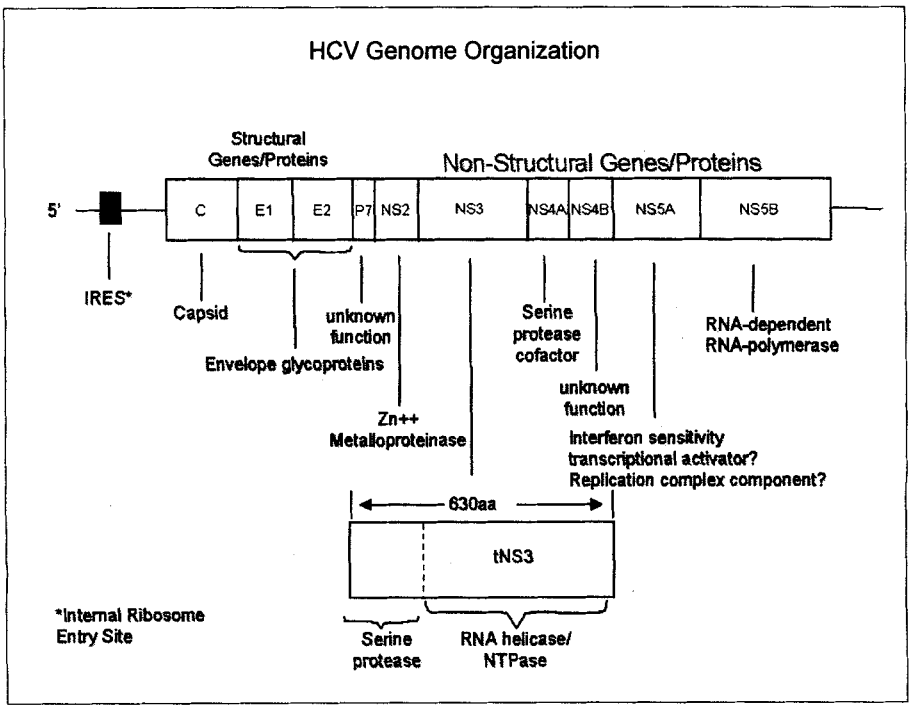
Introduction - Hepatitis C (HCV) is the major causative agent of non-A, non-B hepatitis (1). It is the major cause of transfusion-associated hepatitis and accounts for a significant proportion of hepatitis cases worldwide. Most of the acute infections become chronic, which can lead to an unapparent carrier state with normal or near normal liver functions. Most chronic cases eventually result in liver cirrhosis, which can then lead to liver failure and hepatocellular carcinoma (2). The main route of HCV transmission is parenteral, and the worldwide infection is estimated at several hundred million people. HCV is classified into six major genotypes and over thirty subtypes (3). The low fidelity of HCV's RNA polymerase enzyme results in a high mutation rate, and many different HCV quasispecies are found in clinical isolates.

Although efforts are continuing, there is no vaccine at present (4). The primary treatment currently available for chronic infection is immune modulation by interferon-alpha therapy over 6-12 months. About 40% of treated patients show a decrease of serum alanine aminotransferase to normal levels, although nearly 70% of these responders relapse after cessation of therapy (5). Only about 25% of patients show long-term normalization of serum alanine aminotransferase levels. The beneficial effects of interferon appear to be enhanced when therapy is combined with the purine nucleoside analogue ribavirin, with about 40% of treated patients showing a sustained response. Clearly, there continues to be an urgent medical need for the development of more effective anti-HCV therapeutic agents.



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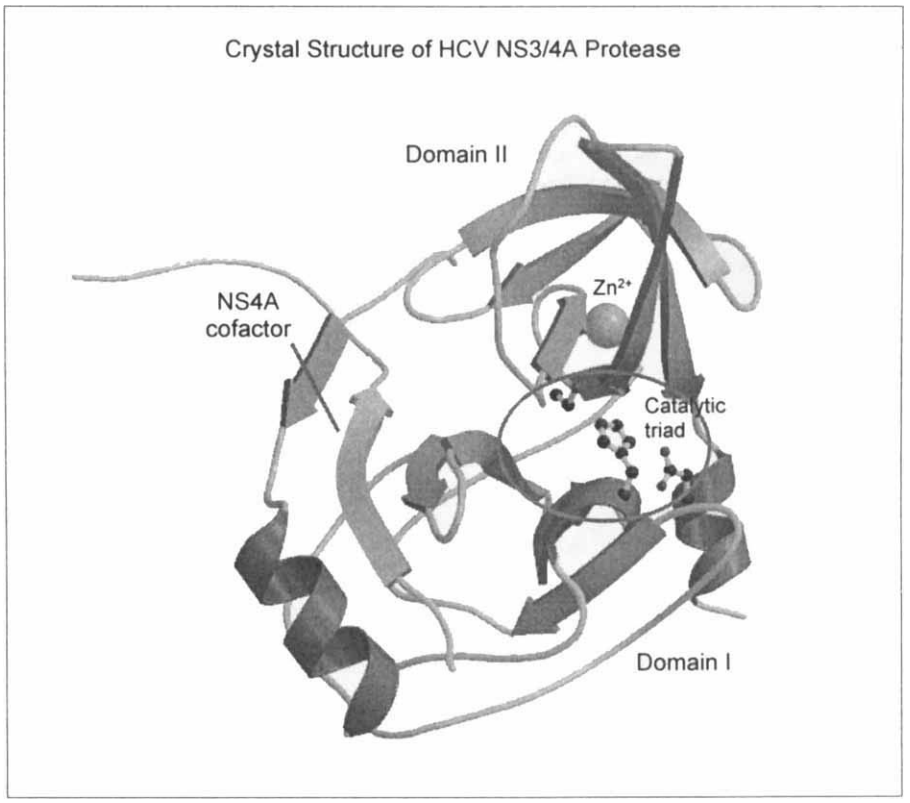
HCV was the first virus to be identified using molecular cloning techniques (6). It is an enveloped virus in the *Flaviviridae* family, with a plus-stranded RNA genome of about 9.6 kb within the nucleocapsid. The genome contains a single open reading frame encoding a polyprotein of just over 3,000 amino acids, which is cleaved co- and post-translationally to generate the mature structural and nonstructural viral proteins. The open reading frame is flanked by 5' and 3' untranslated regions of a few hundred nucleotides in length, which are important for RNA translation and replication. The translated polyprotein contains the structural core (C) and envelope proteins (E1, E2 and p7) at the N-terminus, followed by the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The mature structural proteins are generated via cleavage by the host signal peptidase. The junction between NS2 and NS3 is autocatalytically cleaved by the NS2/NS3 protease, while the four remaining junctions are cleaved by the NS3 serine protease. The NS3 protein also contains the NTP-dependent helicase activity which unwinds duplex RNA during replication. The NS5B protein possesses RNA-dependent RNA polymerase activity. Although the current lack of an efficient system for culturing HCV has precluded definitive proof-of-concept experiments, many of these proteins are considered to be essential for viral replication, and therefore suitable as drug discovery targets in the search for improved antiviral therapeutic agents (5, 7-12).

Serine Protease - Most extensively studied and perhaps most prominent among these HCV targets is the 180-amino acid serine protease contained within the N-terminal third of NS3 (13-15). Validation of this target is currently provided by studies showing that a related flaviviral protease is important for viral replication, and the well-demonstrated precedent that inhibitors of viral proteases can be effective antiviral drugs. NS4A is an important cofactor for cleavage by NS3 protease; *in vitro* studies have shown that NS4A is required for cleavage at NS4B/5A, and enhances the cleavage at NS4A/4B and NS5A/5B.

A number of publications have now reported X-ray crystal structures of this NS3 protease domain with and without a shorter peptide derived from full-length NS4A (16-18). In the crystal structure lacking NS4A, the NS3 protease is folded into two distinct domains, each containing a six-stranded β-barrel similar to other trypsin-like serine proteases. The active site is found in a cleft between these two domains. The serine protease signature triad residues are formed by His-57 and Asp-81 from the N-terminal domain, and Ser-139 from the C-terminal domain. When the NS4A-derived peptide is co-crystallized with NS3 protease, the N-terminal domain of the protease forms an eight-stranded β-barrel similar to the structure of chymotrypsin. The inserted β-strand from the NS4A segment intercalates in an antiparallel fashion between two β-strands of NS3, resulting in a more tightly packed NS3/NS4A structure and bringing the catalytic His, Asp and Ser residues into a more optimized classical triad configuration. Emphasizing the critical role of NS4A, some workers refer to this serine protease as the "NS3/4A protease."

A metal ion binding site is also confirmed by the crystal structure of the NS3 protease. The zinc ion is tetrahedrally coordinated by Cys-97, Cys-99, Cys-145 and His-149 (via a water molecule). Since it is not in the proximity of the catalytic site, this metal ion serves a structural role, being required for the generation of properly folded protein. It has been speculated, however, that this zinc might play a catalytic role for the NS2/NS3 protease which autocatalytically cleaves at NS2/NS3 and thereby exposes the N-terminus of the mature NS3 protein (19).



The substrate binding site of the NS3 serine protease does not display highly prominent features. The binding pocket is long and relatively shallow in nature. It is fairly solvent exposed and non-polar in content. Biochemical studies have noted the requirement of comparatively long peptides for effective substrates, and a minimal decapeptide spanning P6-P4' is commonly used. The distinguishing feature of the binding site is the S1 subsite, which is a small hydrophobic pocket defined by Phe-154, Ala-157 and Ile-135. Preferred substrates contain the P1 cysteine which exerts favorable electrostatic interaction with the Phe-154 residue. Cysteine at P1 is conserved at all three *trans*-cleavage sites, while threonine is found at P1 of the *cis* (NS3/NS4A) cleavage site. Serine at P1' is also highly conserved, and alanine at P1' is found only in some strains at the NS4A/NS4B cleavage site. Of particular note is an acidic residue (aspartic acid or glutamic acid) found to be highly conserved at the P6 position of all these sites.

Conceptually, strategies for inhibition of NS3 protease activity suggest three possible target sites based on knowledge of structure and mechanism: active site, NS4A binding site, and metal ion binding site. To date, only one inhibitor of NS4A binding has been reported, an NS4A peptide analog with an *in vitro* IC_{50} value of 20 μM (20). Emerging studies of full-length NS3/NS4A suggest a very high affinity for this interaction (21). Coupled with the rather formidable effective concentration of NS4A in the vicinity of NS3 required for this *cis* cleavage, these results suggest that it may be especially difficult to find an effective small molecule inhibitor based on this approach. The possibility of identifying inhibitors based on chelation of the structural zinc has been described (22); rigorous specificity may be necessary to avoid toxicity in a cellular milieu.

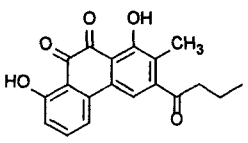
Classical active site-directed, commercially available, small molecule serine protease inhibitors are rather ineffective against NS3 protease. High concentrations of chymotrypsin-like inhibitors such as N-tosyl-L-phenylalanine chloromethylketone, chymostatin and Pefabloc SC, and the general serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride are required for inhibition of NS3 protease (23-26). Other classical serine protease inhibitors such as diisopropyl fluorophosphate, 3,4-dichloroisocoumarin, aprotinin, antipain, leupeptin, and N-α-p-tosyl-L-lysine chloromethyl ketone show essentially no inhibition of NS3 protease (23, 24, 27, 28). The inefficient binding of these small molecule inhibitors is likely due to the uncommonly shallow and relatively non-polar substrate binding pocket of NS3 protease. These properties present a significant challenge for structure-based design approaches.

Extensive studies of substrate sequence requirement, preference, and modification have provided valuable information for the design of potent peptidic inhibitors directed to the active site of NS3 protease. The non-cleavable decapeptide Glu-Asp-Val-Val-Leu-Cys-Tic-Nle-Ser-Tyr, which was modelled after the NS5A/NS5B cleavage site, has a Ki value of 340 nM (29). Independent design of other potent peptide inhibitors of NS3 protease was based upon the observation of product inhibition by the carboxylic acid components. Hexapeptidic carboxylic acids based on cleavage site sequences were shown to be substrate-competitive inhibitors. These peptides (Ac-DEMEEC-OH, Ac-EDVVCC-OH and Ac-DCSTPC-OH), which were modelled after the NS4A/NS4B, NS5A/NS5B and NS4B/NS5A cleavage sites, showed Ki values of 0.6, 1.4 and 180 μM, respectively (30). A series of residue optimizations, which utilized the power of combinatorial chemistry, led to the identification of even more potent hexapeptide inhibitors. The most active compound reported, Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH (where Gla is γ-carboxyglutamic acid and Cha is cyclohexylalanine), inhibited NS3 protease with an IC_{50} value of 1.5 nM (31).

Electrophilic functionalities at the P1 residue based upon this product inhibition were also examined by several researchers. In the hexapeptide template where Ac-DDIVPC-OH was noted as a moderate inhibitor of NS3 protease with an IC_{50} value of 28 μM, it was also observed that a D-amino acid residue at the P5 position resulted in a significant enhancement of inhibitory activity. Ac-DdIVPC-OH (where small case letter indicates D-amino acid) inhibited NS3 protease with an IC_{50} value of 4 μM (32). Using Ac-DdIVPNva-OH (where Nva is norvaline) as a reference P1 carboxylic acid inhibitor (with IC_{50} value of 17 μM), the corresponding P1 aldehyde, trifluoromethyl ketone, pentafluoropropylketone and benzylketoamide showed NS3 protease inhibitory activities with IC_{50} values of 1.1, 22, 12 and 0.64 μM, respectively (33). It was interesting to note that the P1 carboxylic acid inhibitor showed no inhibitory activity (IC_{50} > 1 mM) against human leukocyte elastase, procine pancreatic elastase, bovine pancreatic α-chymotrypsin and human liver cathepsin B. The other peptidic inhibitors with electrophilic functionalities at P1 residue, however, showed high inhibitory activities against the other proteases, with many inhibiting the elastases with near nanomolar potency.

Screening of random compound collections has identified additional inhibitors of NS3 protease; none of these are reported to be active site-directed or competitive with substrate. A phenanthrenequinone (1), which is a secondary metabolite from *Streptomyces* sp., was reported to show an IC_{50} of 2.5 μg/mL (34); the mechanism of inhibition has not been reported. A series of 2,4,6-trihydroxy-3-nitrobenzamides were reported to show inhibitory activity (35). While compound **2** was observed to be the most active compound, with an IC_{50} value of 2.3 μg/mL,

compound **3** (with an IC_{50} value of 32.8 μg/mL) was shown to be selective against chymotrypsin and elastase (IC_{50} values above 100 μg/mL). Kinetic study with compound **4** (IC_{50} value of 10.1 μg/mL) concluded that the mode of inhibition was a mixture of noncompetitive and uncompetitive inhibition with Ki values of 11.4 (for NS3/NS4A alone) and 6.9 μM (for NS3/NS4A with substrate). A series of thiazolidines have also been identified as HCV protease inhibitors (36). Compound **5** was the most active compound in the group with an IC_{50} value of 2.3 μg/mL. Compound **6** (with an IC_{50} value of 6.4 μg/mL) showed selectivity against chymotrypsin (IC_{50} value of 15.6 μg/mL) and trypsin (IC_{50} value of 18.3 μg/mL) and did not inhibit plasmin or elastase (IC_{50} values above 50 μg/mL). Kinetic study with compound **6** showed it to be a noncompetitive inhibitor with Ki values of 5.3 (NS3/NS4A alone) and 5.5 μM (NS3/NS4A with substrate). Screening of a random collection of another 2,000 compounds identified a number of NS3 protease inhibitors including compounds **7**, **8** and **9** with IC_{50} values of 3.2, 6.5 and 6.2 μM, respectively (37). The latter two compounds were shown to be noncompetitive inhibitors with Ki values of 2.4 and 3.6 μM, respectively.



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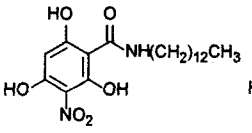

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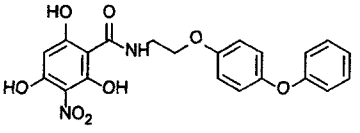

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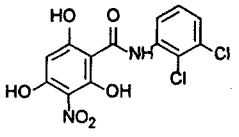

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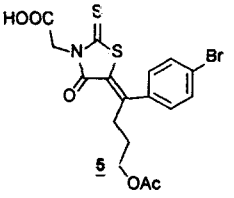

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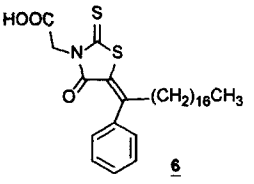

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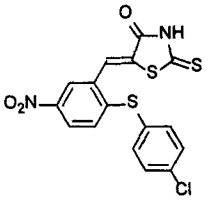

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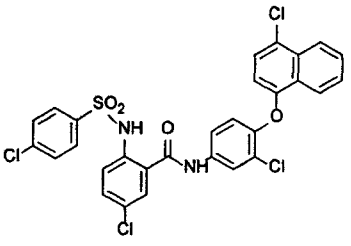

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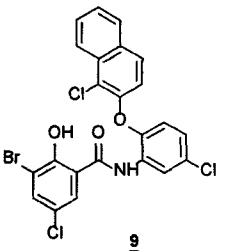

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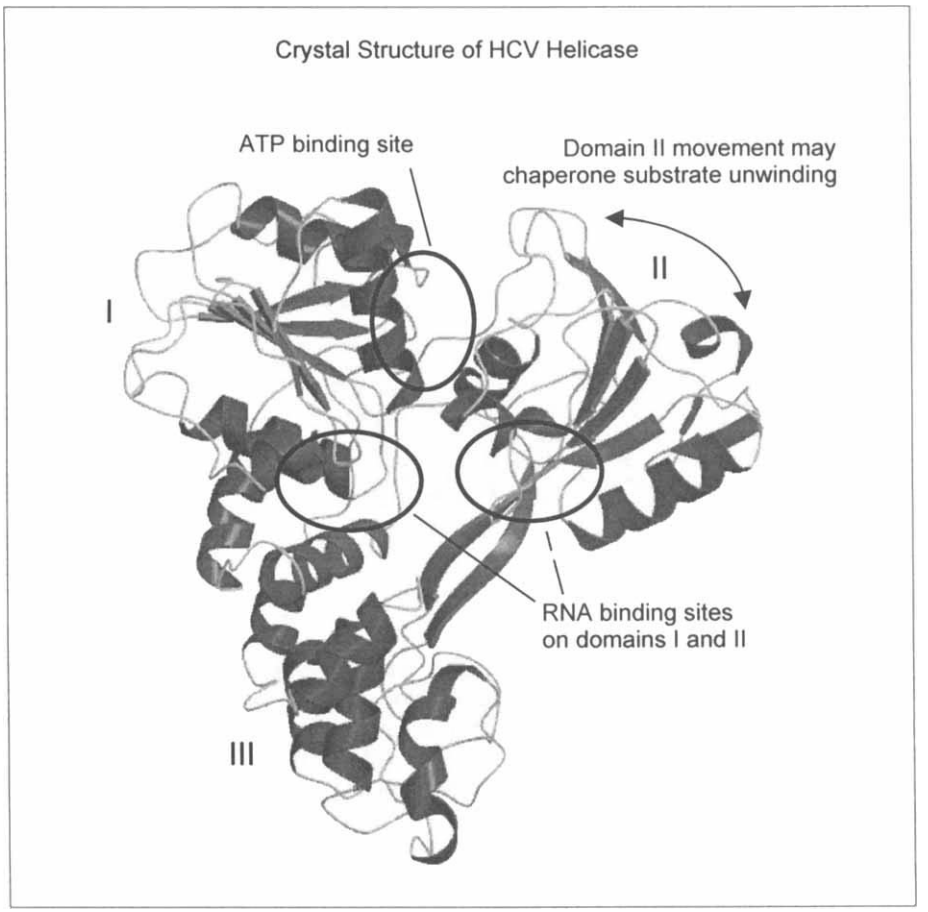
In addition to these small molecule approaches for inhibition of NS3 protease, several protein- and nucleic acid-based inhibitors have been reported. One approach is based on remodeling the serpin Eglin C (38). Although this 70-amino acid potent inhibitor of several serine proteases did not inhibit NS3 protease at a concentration as high as 180 μM, re-engineering of its binding loop based upon the sequences of cleavage sites recognized by NS3 protease resulted in a modified eglin with a Ki of 30 nM. The same research group has also identified antibody-based inhibitors as a result of screening phage-displayed libraries of minimized antibody domains; the best of these inhibitors has a Ki of 360 nM (39). Finally, there

have been several reports of RNA aptamer inhibitors, e.g., a 120-base pair RNA with a Ki of 3 μM (40).

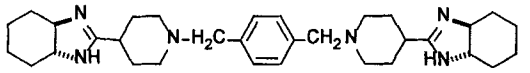
RNA Helicase - The C-terminal two-thirds of NS3 contains the NTPase/helicase functions and constitutes another rational target for drug discovery (41). *In vitro* studies have shown that unwinding of a duplex RNA substrate is coupled to NTP hydrolysis. Although no helicase inhibitors of any kind currently exist as marketed drugs, recent studies with vaccinia virus have demonstrated the importance of these enzymatic activities for viral replication (42).

A number of publications have described the X-ray crystal structures of the HCV helicase domain (43-45). The NTPase/helicase is folded into three distinct domains which together form a Y-shaped triangular molecule. The first and second domains possess the βαβ structures, which are alpha helices packed around a central beta sheet. The third domain consists primarily of alpha helices. The first and third domains are closely connected and form a rigid unit, while the second domain is separated by a distinct groove from other two. The second domain appears to have the flexibility to rotate as a rigid body and can pivot relative to the first and third domains. The interdomain cleft constitutes a part of the helicase active site. The residues which are required for RNA unwinding are located in the arginine-rich conserved region in the second domain. In the crystal structure of HCV



nelicase complexed with the poly dU8 oligonucleotide, the single stranded DNA lies in a channel that separates the third domain from the first and second domain (44). The 5'-end of the polynucleotide lies at the interface of the second and third domains, while the 3'-end lies at the interface of the first and third domains. The transmission of conformational change likely occurs as a consequence of NTP hydrolysis as the substrate is unwound. The NTP binding site is located in the helicase first domain, which displays conserved NTP binding site residues within the nucleotide binding fold. The Walker motif A binds the terminal phosphate group of the NTP cofactor, while the Walker motif B chelates the essential magnesium ion of the Mg-NTP complex. Effective inhibition of HCV helicase could therefore result from binding at the oligonucleotide binding site, the NTP binding site, or perhaps an allosteric site critical for the dynamic movements envisioned for this enzyme.

Few small molecule inhibitors of HCV helicase have been reported to date. The nucleotide analogues adenylyl-(β,γ-methylene)-diphosphate, adenylyl-imidodiphosphate and adenosine 5'-O-(3-thiotriphosphate) inhibit the helicase activity with increasing potency (46). Compound **10** inhibits HCV helicase activity with an IC_{50} value of 7 μM, while compound **11** has an IC_{50} value of 10 μM (47, 48). Interestingly, a series of compounds with spacers of variable length (compounds **12**, where n = 2,4,6,7,8) exhibit the same inhibitory activity with an IC_{50} value of 0.7 μM (48).



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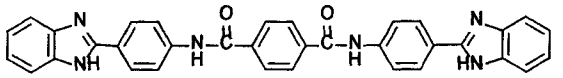

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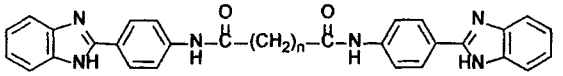

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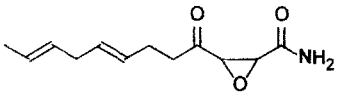
RNA Polymerase - Another HCV enzyme target is the RNA-dependent RNA polymerase encoded by NS5B, considered essential for viral replication (49-51). Further validation for this target comes from the discovery and development of small molecule drugs directed against other viral polymerases. After expressing and purifying recombinant HCV NS5B, several groups have begun the characterization of this enzyme and developed *in vitro* activity assays suitable for inhibitor screening. Although NS5B alone is typically used in these assays, it is expected that additional proteins derived from the host and/or virus will enhance the activity, processivity, and specificity, as envisioned within the context of a viral replication complex. In this regard, interactions like the one recently identified between NS5B and NS3 may also be suitable targets for therapeutic intervention (52). Several weak, small molecule inhibitors of NS5B polymerase activity have been reported recently: a few nucleoside 5'-triphosphate substrate analogs, plus the natural products cerulenin (**13**) (IC_{50} ~ 500 μM) and gliotoxin (**14**), the latter shown to be a mixed noncompetitive inhibitor with a Ki of 230 μM (50, 51).



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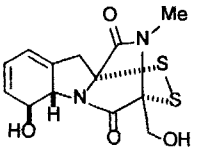
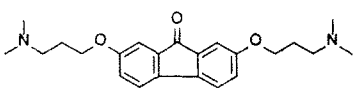


14

5'UTR and IRES - In addition to these enzymatic targets, the 5' UTR (untranslated region) of HCV contains a structural target. The secondary structure of the RNA in this region constitutes the IRES (internal ribosome entry site) required for protein translation (53). Antisense oligonucleotides, "decoy" RNA and hammerhead ribozymes directed against this IRES inhibit protein synthesis *in vitro* (54-56). Also, several groups have now reported the identification of small molecule inhibitors from screening studies. Compounds such as the fluorenone **15**, the xanthone **16** and compound **17**, related to ellagic acid, were reported to show inhibition of IRES-dependent translation *in vitro* (57, 58).



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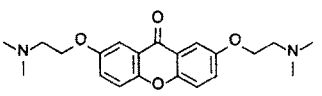

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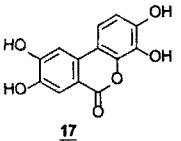

17

Other Targets - Another HCV structural target is presented by the recent identification of CD81 as a putative cellular receptor for HCV (59). The HCV envelope protein E2, as well as HCV infectious plasma itself, were shown to bind human CD81, a membrane-spanning protein expressed on cell types known to be infectable by HCV. This *in vitro* binding, moreover, was inhibited by anti-E2 antibodies which neutralize HCV infection *in vivo*. Although E2/CD81 is clearly a protein/protein interaction, these experiments raise the possibility that small molecule inhibitors might be discovered with the same neutralizing effect. Also, unlike the other HCV targets considered here, this one may offer some advantages in being extracellular. With HIV, an analogous approach has been taken in targeting the interaction between viral coat proteins like gp120 and cellular receptors like CD4 and CCR5. Although coreceptors for HCV have not yet been identified, a recent study suggests that the LDL receptor may also be involved in the uptake of HCV (60).

HCV encodes a variety of other functions which are known or expected to be important for viral replication and persistence (5, 11, 12). These interactions, some of which involve both viral and host proteins, present additional opportunities for novel small molecule intervention. NS5A contains an "interferon sensitivity determining region" (ISDR) which has been shown to interact with a cellular protein kinase and may be responsible for mitigating the response to interferon treatment among some individuals. In addition to the NS3/NS5B interaction and 5'UTR interactions mentioned above, HCV's 3'UTR has been shown to interact with the host protein pyrimidine tract binding protein (PTB). The protease activity associated with NS2/3 is another possible target, although the single cleavage it catalyzes, thought to occur in *cis*, may be difficult to inhibit (19).

It is expected that ongoing research will identify other suitable targets and approaches, some of which may not directly involve HCV-encoded activities. For

example, phase II clinical trials have recently been initiated for HCV therapy with a small molecule inhibitor of inosine monophosphate dehydrogenase (IMPDH), a host enzyme important for *de novo* nucleic acid synthesis. Other recent, novel small molecule approaches for HCV treatment include the use of imino sugars as protein folding inhibitors; new immune response modifiers; and HCV inactivation systems for blood platelets.

Concluding Remarks - Discovery and optimization of small molecule HCV antivirals would be greatly aided by an efficient cell culture system for propagation of the virus. Although there have been recent, encouraging reports along these lines, the viral titers in these systems have been low, mirroring the relatively low virus titer in infectious sera and the low expression levels of viral antigens in infected tissues (11). In the meantime, cell-based assays recently developed for at least one HCV target, NS3 protease, allow researchers to bridge the gap between *in vitro* screening assays and animal models like the chimpanzee or a more recently developed mouse system (13, 14, 61).

As with HIV therapies, resistance to any one HCV target-directed therapy is expected to develop rapidly. As mentioned above, HCV polymerase has a high error rate, resulting in a large number of quasispecies present within any given individual at one time. It is therefore expected that combination therapy, both in terms of agents directed against multiple targets and multiple agents directed against any given target, will be necessary for effective control of HCV infection. Clearly, there are multiple opportunities for the discovery and development of such antivirals.

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Chapter 14. Antimitotic Agents

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Introduction - Cancer is projected to become the number one cause of death in industrialized nations by the year 2000. The market for cancer chemotherapy was estimated at about 10 billion dollars worldwide in 1997 and continues to expand at a double digit annual growth rate. Truly selective cancer chemotherapy, in which a drug will specifically destroy malignant tumor cells, is the ultimate goal of cancer research. Emerging science within the past decade has created opportunities for fundamentally new ways of approaching the disease (1). Biochemical processes in the cell cycle that are unique to cancer cells have become new targets in cancer research (2-4). Despite the promise of cytostatic agents that are selective for these processes, cytotoxic drugs will remain a mainstay in cancer chemotherapy for the near future (5-6).

Antimitotic agents constitute a major class of cytotoxic drugs and have been the subject of several reviews (7-11). Microtubules, which are the dynamic pipe-like protein fibers composed of alternating α- and β-tubulins, are integral components of the mitotic spindle which is intimately involved in cell replication. By interfering with the natural dynamics of tubulin polymerization or depolymerization, the antimitotic agents inhibit cell proliferation by arresting dividing cells in metaphase. Tubulin binding molecules have generated considerable interest among cytotoxic agents, due in part to the successful introduction of the taxanes into clinical oncology and to the widespread use of the vinca alkaloids (7,12-14)). However, the development of resistance to these agents has limited their ultimate effectiveness. Although membrane P-glycoprotein mediated multidrug-resistance (MDR) is known to exist among taxanes and vinca alkaloids (15-16), differential expression of various tubulin isotypes has also been implicated in resistance to taxanes and other antimicrotubule agents (17-19). Renewed interest in antimitotic agents has been generated by the hope that non-MDR substrates that interact with tubulin at sites different from those of vinca alkaloids and taxanes can be discovered. There are three major classes of antimitotic agents. These classes are microtubule stabilizing compounds, vinca alkaloid site binders, and colchicine-site binders (CSB). The former class of compounds inhibits the microtubule depolymerization by binding to and stabilizing microtubules, while the latter two classes inhibit tubulin polymerization into microtubules.

The 3.8 Å structure of the tubulin α/β heterodimer, which assembles into the microtubules of the mitotic spindle, has recently been solved by electron crystallography of zinc-induced tubulin sheets (20-21). A molecular model which includes the binding sites of paclitaxel, colchicine and GTP has been proposed by integrating the crystal structure and biochemical data. Both colchicine and paclitaxel bind on the side of tubulin that faces the lumen of the microtubule, with colchicine near the α–β interface and paclitaxel bound within the β-monomer (22). Possible relative locations of colchicine, paclitaxel and GTP on tubulin have also been probed using fluorescence spectroscopy (23).

Microtubule stabilizing compounds - In contrast to the CSBs and vinca alkaloids, microtubule-stabilizing agents, exemplified by the taxanes, stabilize microtubules

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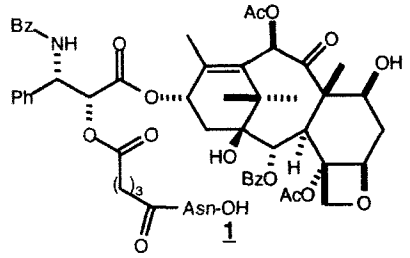
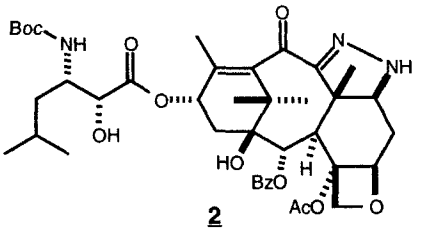
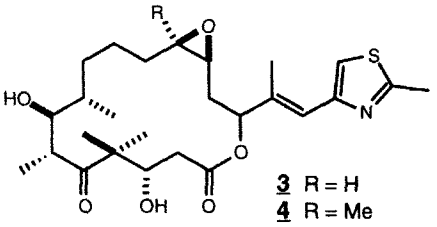
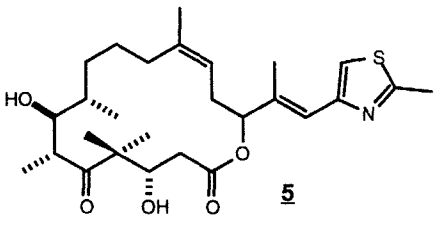
and block their depolymerization (24). Despite these differences, the consequence of disruption of tubulin and microtubule dynamics appears to be the same: metaphase arrest in dividing cells and induction of apoptosis. The biology and SAR of the taxanes have been reviewed (12-14). Design of recent structures has been directed towards solving the limitations of both solubility and resistance. One approach has been through the use of prodrugs. Compound **1** is > 1500-fold more soluble than paclitaxel and possesses similar cellular potency, suggesting ester cleavage *via* lysosomal enzymes (25). A poly(*L*-glutamic acid)-paclitaxel conjugate has been shown to be both more water soluble than paclitaxel and to possess an improved therapeutic window in animal models (26). Compound **2** is an example of a modified taxane with improved activity against cells with the MDR phenotype (160-fold resistant vs. 1100-fold resistant for paclitaxel) (27).

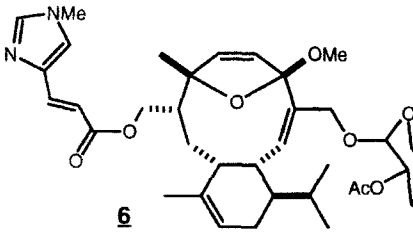


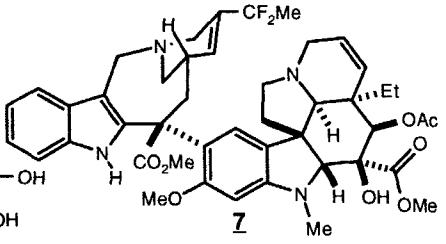






Over the last several years, a number of other microtubule-stabilizing natural products have been discovered. The most well characterized are the epothilones (**3**, **4**, reviewed in 28). While competitive binding experiments suggest that epothilones occupy the same site on microtubules as the taxanes, resistance to them is not induced by the MDR phenotype and they are active against a number of taxol-resistant cell lines. *In vivo* characterization of derivative **5** indicates that it is less toxic than **4** and more efficacious than paclitaxel in an MX-1 human mammary carcinoma xenograft model (29). Eleutherobin **6** (and the related aglycone sarcodictyin A) also appears to bind to the taxane site on microtubules, but unlike epothilone, these latter compounds are cross-resistant with paclitaxel (30).

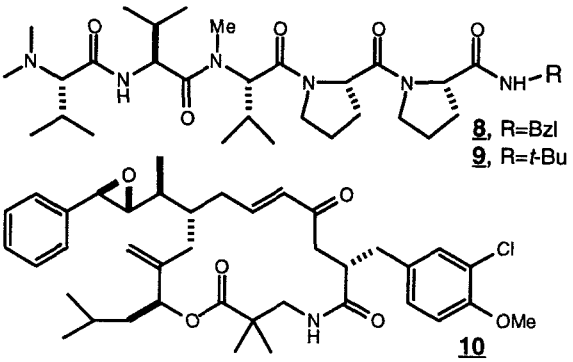




Vinca alkaloid site binders - Vinca alkaloids, as represented by vinblastine and vincristine, prevent microtubule assembly by binding to tubulin at a site distinct from colchicine. Despite their high toxicity, vinca alkaloids are used extensively. The

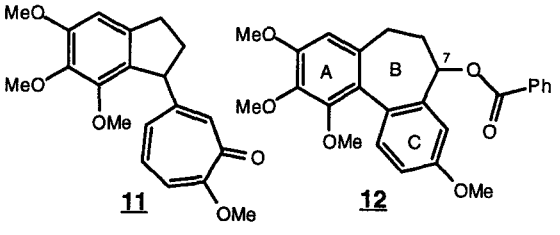
SAR, clinical usage, and pharmacology of vinca alkaloids have been reviewed recently (7,9). The newer, semisynthetic agent vinorelbine appears to have reduced neurotoxicity and has been used to treat a variety of human tumors (9,31). Further modification of vinorelbine in superacidic media led to the discovery of vinflunine (7), which demonstrated significantly superior antitumor efficacy *in vivo* as compared to vinorelbine in each tumor model evaluated (32-33).

Other compounds sharing a similar mechanism of action to the vinca alkaloids have been reviewed (8-10). One of the most promising compounds in this class is cemadotin (LU103793, 8), which is a water soluble pentapetide analog of dolastatin 15 and is currently in phase II clinical trials for metastatic melanoma and other solid tumors (34-35). Its primary mode of action remains unclear but is believed to be similar to that of vinca alkaloids (36). Cemadotin shows good activity against a broad range of murine and human tumors, with IC_{50} values typically in the picomolar range (37). Cemadotin shows excellent efficacy in a number of xenograft and synegeneic tumor models (11). Compound 9 (LU223651) is the latest in this series and is orally bioavailable with similar *in vivo* activity and slightly reduced *in vitro* activity as compared to cemadotin (38). Cryptophycin 52 (LY355703, 10) is a new member of the cryptophycin family. It has demonstrated potent antiproliferative activity in both solid and hematologic tumor cell lines including MDR phenotypes, with IC_{50} values in the low picomolar range, significantly lower than paclitaxel and vinblastine (39). It inhibits microtubule polymerization and depolymerizes preformed microtubules by high-affinity binding to tubulin, and arrests cells in the G2/M phase of the cell cycle at lower concentrations (40).



10

Colchicine-site binders (CSB) - The study of colchicine has played an important role in our understanding of drug-tubulin interactions. As the first member of this class of inhibitors, colchicine itself is very toxic and fails to show any efficacy *in vivo* at the maximum tolerated dose (MTD). The key challenges here, and for CSBs in general, are to generate compounds with potent *in vivo* efficacy and good safety margins. Studies of colchicine analogs in attempts to produce useful compounds with simplified structures and reduced toxicity have thus far proved unfruitful. The SAR of colchicine analogs has been reviewed recently (9). The trimethoxyphenyl group of the A-ring of colchicine serves as a complex-stabilizing anchor on tubulin in the inhibition of microtubule assembly and is essential for its activity (41).

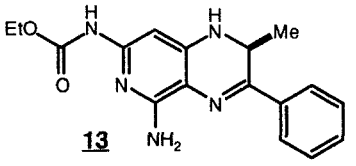


11 12

A number of colchicine analogs have been published recently. A modification on the B-ring of colchicine resulted in simple analog 11, which displays almost

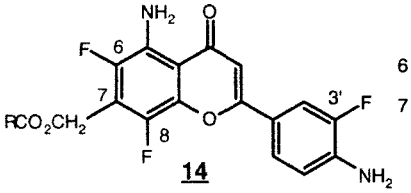
identical activity in inhibition of tubulin polymerization, but the cellular cytotoxicity (GI_{50}) is much higher, ranging from 0.1 to 10 μM (42). Interestingly, the inhibition of tubulin polymerization by **11** is temperature dependent, being nearly 6-fold more active at 37 °C than that at 30 °C. The 7-O-substituted analog of deaminodeoxy colchinol, **12**, inhibits tubulin polymerization with an IC_{50} of 2.7 μM (43). The (7*R*)-isomer of **12** is more active than the (7*S*)-isomer, which is opposite the trend observed for colchicine and its analogs.

The phenyl dihydropyridopyrazine CI 980, **13**, inhibits tubulin polymerization at the colchicine site and demonstrates excellent *in vitro* activity against a variety of tumor cell lines, including MDR phenotypes, in the nanomolar range (10-11). Phase II clinical studies of CI 980 have been published recently (44-45).

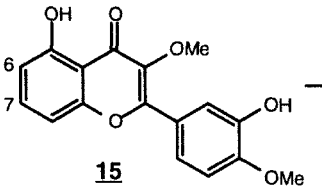


13

Certain flavonoids are known to possess antitumor activity (46). A series of 7-substituted-diaminofluoroflavones (**14**) was found to be potent CSBs and to have *in vitro* cytotoxicity against the MCF-7 cell line with IC_{50}s in the nanomolar range (47). Good *in vivo* antitumor activity of **14** (R=*n*-pentyl) was observed (6.3 to 25 mg/kg) against MCF-7 in nude mice, with T/C(%) ranging from 27 to 0.8. A series of flavones that are structurally related to centaureidin (**15a**) has been screened for cytotoxicity in the NCI *in vitro* 60-cell line human tumor screen (48). Out of the 79 compounds, compounds **15b-c** have IC_{50}s for tubulin polymerization of 0.83 and 3.0 μM, respectively. They also show significant cytotoxicity (GI_{50}<2 μM). These three compounds inhibit the binding of colchicine to tubulin with IC_{50}s ≥ 5 μM.



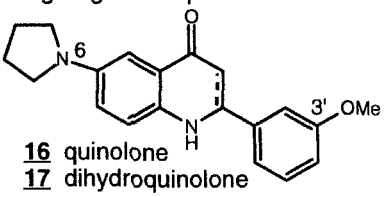
14



15

	C-6	C-7
a	OMe	OH
b	OMe	OMe
c	H	OH

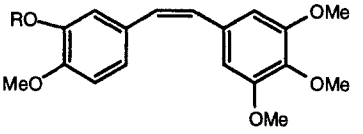
Further research in this area resulted in the discovery of a series of 2-phenyl-4-quinolones and 2-phenyl-2,3-dihydro-4-quinolones (**16**, **17**), which are potent CSBs comparable to colchicine (9). The 2,3-dihydro-4-quinolones are generally more potent than the corresponding 4-quinolones. The 3'-methoxy or chloro group is essential for their activity. The 6-pyrrolidinyl analog (**17**) is the best compound in this series, with cellular potency in the nanomolar range against a panel of human tumor cell lines, and with an antitubulin IC_{50} of 0.66 μM (49). Generally, the (*2S*)-isomers are several fold more potent than the (*2R*)-isomers. Their cytotoxicity and antitubulin activity are closely correlated. No *in vivo* efficacy data of this class of compounds has been reported yet.



16 quinolone
17 dihydroquinolone

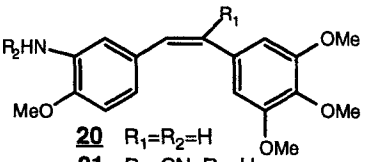
Combretastatin A4 (CA-4, **18**) is one of the most potent antimitotic agents that interacts with the colchicine binding site, yielding an IC_{50} of 0.53 μM *versus* tubulin polymerization (49). Research on CA-4 has centered on improving the water solubility and *in vivo* efficacy. In an effort to increase aqueous solubility, the water-soluble disodium phosphate prodrug **19** of CA-4 was prepared (50-51). CA-4 and **19**

display potent and selective antiangiogenic activity (52-55). CA-4 induced vascular shutdown within experimental tumor models *in vivo*, with a reduction in functional vascular volume of 93% at 6 hr, and persisted over 12 h following i.p. administration of the drug at doses less than one-tenth of the MTD (52). CA-4 reduced tumor energetics and pH, as determined by ^{13}P and ^{1}H NMR, which is consistent with a reduction in tumor blood flow (56-57). CA-4 is markedly more effective *in vivo* when combined with radiation therapy than the radiation therapy alone (53). However, CA-4 fails to show any efficacy *in vivo* against murine colon 26 adenocarcinoma (58). This lack of efficacy *in vivo* is believed, in part, to be due to its poor solubility and pharmacokinetic properties.



18 R=H; **19** R=PO_3Na_2

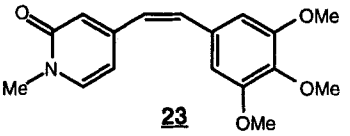
Perhaps one of the most important findings among combretastatin-related analogs was the discovery that replacement of the phenolic hydroxy group in CA-4 with an amino group leads to marked increases in water solubility and in efficacy *in vivo*. The water soluble amino stilbene analog **20** (AC-7739) and the cyano analog **21** (AC-7728) display IC_{50}s of 5.9 nM or less against murine colon 26 adenocarcinoma, which represents a more than 3-fold increase in cytotoxicity over CA-4 (58). The tubulin polymerization inhibition of both AC-7739 and AC-7728 are identical or slightly less active in comparison to CA-4. *In vivo*, compounds AC-7739 and AC-7728 achieved T/C ranging from 23 to 41% *versus* murine colon 26, colon 38 and 3LL in mice when administered intravenously at MTD, as compared with up to 69% for CA-4 and cisplatin. AC-7739 appears to be superior to AC-7728 and cisplatin *versus* HCT-15 human xenografts in nude mice. The MTD of AC-7739 is 4-fold higher than that of AC-7728.



20 R_1=R_2=H
21 R_1=CN, R_2=H
22 R_1=H, R_2=Ser

In an effort to further improve water solubility and *in vivo* efficacy of AC-7739, the *N*-serine prodrug **22** (AC-7700) was prepared and was observed to be converted readily to the parent AC-7739 in the presence of plasma (59). The *in vivo* efficacy of AC-7700 *versus* colon 26 is superior to AC-7739, achieving T/C of 18% at an iv dosage of 80 mg/kg, as compared with 31% at MTD (40 mg/kg) for AC-7739.

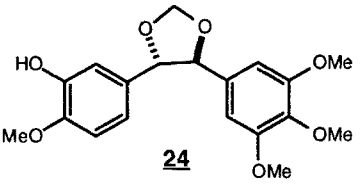
A number of B-ring modified CA-4 analogs have been reported (60). In general, replacing the phenyl ring with other aromatic heterocycles leads to sharply decreased activity, particularly in cell cultures. The best compound prepared was the *N*-methylpyridone analog **23**, which is only 2-fold less potent than CA-4 *versus* colon 26. The solubility of **23** in plasma is improved to 5.2 mg/mL, compared with 1.5 mg/mL for AC-7739.



23

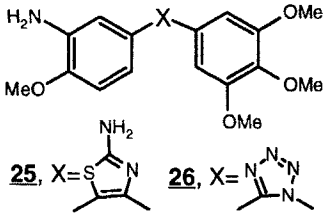
The structural features of CSBs consist of two substituted phenyl rings joined together by a linker. The 3,4,5-trimethoxyphenyl group is essential in most cases for activity. Linkers that allow the two phenyl rings to be located *cis* to each other, such as *cis*-double bond, were found to be optimal (61). SARs of a series of conformationally constrained dioxolane based analogs in which the two phenyl groups occupy the 4,5-positions were reported (62). The (*S, S*)-isomer (**24**) was found be the most potent compound prepared. Its antitubulin IC_{50} is comparable to that of CA-4. Surprisingly, the other three isomers of **24**, including the (*R, R*)- and

the *cis* compounds, are much less active against tubulin polymerization, with IC_{50}s > 50 μM. Compound **24** shows a GI_{50} of less than 5.2 nM against the multidrug resistant and P-glycoprotein expressing PC-12 cell line, which was 20 and 60 times more potent than vincristine and cisplatin, respectively.

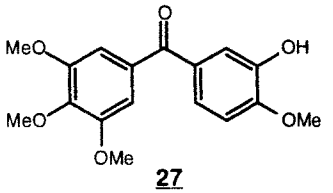


24

Analogous to the dioxolane analogs, thiazole and tetrazole were also used as linkers in place of the double bond (63). Aminothiazole analog (**25**) shows an IC_{50} of 57.5 nM against colon 26 and is 8-fold less potent than the tetrazole analog (**26**). Compounds **25** and **26** are superior to CA-4 in inhibition of tubulin polymerization. Compound **25** is well tolerated in mice with MTD up to 160 mg/kg (sc), compared with 40 mg (iv) for **25** and AC-7739. In animal tests against colon 26, T/Cs were 25% and 11% for **25** (iv) and **26** (sc), respectively, following dosing at the MTDs, compared with 27% for AC-7739 and 36% for cisplatin.



Phenstatin (**27**), in which a carbonyl group was used as a linker for the two phenyl groups in CA-4, is a potent inhibitor of tubulin polymerization, comparable to CA-4 (64). A series of diaryl compounds (**28**, **29**) in which the carbon-carbon double bond of CA-4 was replaced with an oxygen atom, a nitrogen atom and a methylene group as linkers was synthesized and tested for antimitotic activity (42,65). None of these compounds are as potent as CA-4. The most potent compound **29** has GI_{50} values ranging from 0.1-10 μM and IC_{50} of 11.2 μM against tubulin polymerization.



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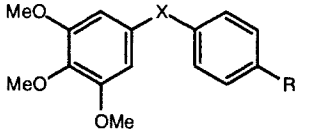
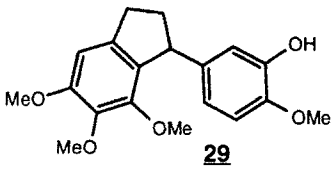


28 X=O, NAc, NH
R=H, Me, Et, OMe

A thyroid hormone analog (oncocidin A1, **30**) was reported to inhibit microtubule assembly by binding to the colchicine site on tubulin (66). It has demonstrated notable antitumor effects against breast and ovarian carcinoma xenografts when given orally at 500 mg/kg BID, and it represents another interesting series of antimitotic agents with structural features distinct from combretastatin.



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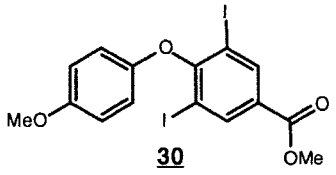
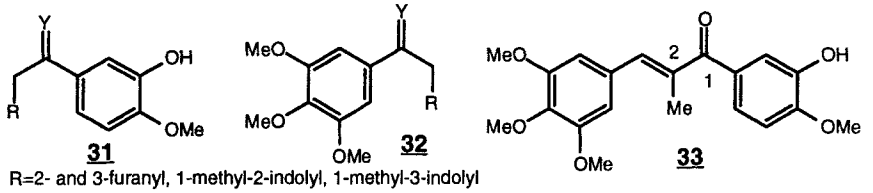


30

Two series of compounds resulting from the formal replacement of either the trimethoxyphenyl (**31**) or the 3-hydroxy-4-methoxyphenyl (**32**) groups of CA-4 by heteroaromatic groups have been identified as CSBs (67). The most potent analog (**32**, Y=O and R =1-methyl-2-indolyl) has cellular IC_{50}s ranging from 0.3 - 0.6 μM against a panel of cell lines including P388, A-549, HT-29 and MEL-28.

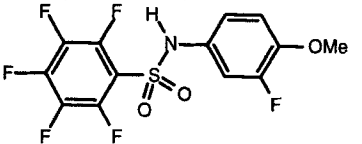
The α-methyl-substituted chalcone (**33**), which has a three-carbon linker, was reported to have potent anti-mitotic and cell growth inhibitory properties, demonstrating an IC_{50} of 0.21 μM against the K562 cell line in the MTT assay (68).



31 **32** **33**

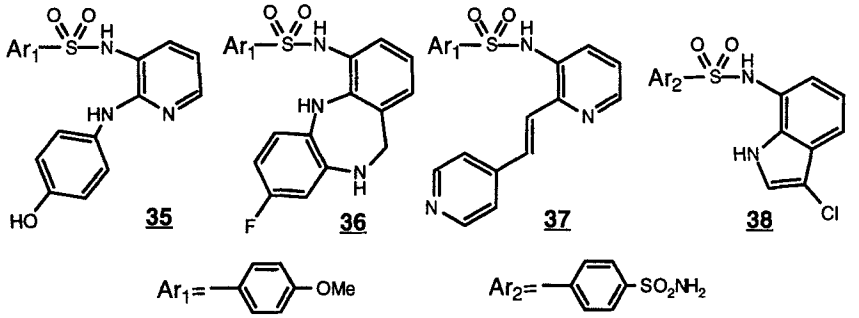
R=2- and 3-furanyl, 1-methyl-2-indolyl, 1-methyl-3-indolyl

In a major departure from the CA-4 structure motif, a series of orally active pentafluorobenzenesulfonamides, exemplified by **34** (T138067), was found to bind at the CSB site irreversibly to β-tubulin (69). Removal of two fluorine atoms in T138067 resulted in at least a 2-log decrease in cytotoxicity, suggesting that the pentafluorophenyl motif is requisite for the activity of this series. T138067, which was the best compound in this series, has been shown to inhibit the growth of a variety of human tumor cell lines including MDR phenotypes. Compared to paclitaxel and vinblastine, T138067 displayed superior efficacy against MX-1 human mammary tumor and similar activity against CCRF-CEM lymphoblastic leukemia xenografts in mice. The *in vivo* activity was not affected by MDR+ cell lines (70).



34

Another promising series of sulfonamide compounds is represented by **35**, **36**, and **37**. It appears that a substituted arylamino or aroylamino group *ortho* to the sulfonamide bond is critical to maintain activity. The *para*-methoxybenzenesulfonamide was found to be optimal. An early compound in this series, E7010 (**35**), inhibits tubulin polymerization in the M phase of mitosis by binding at the colchicine site (17,71-72). E7010 is quite soluble in water as an acid salt. It is well absorbed orally with bioavailabilities ranging from 25 to 92% in different animal species (73). The plasma concentration-time profile suggested possible enterohepatic recycling. E7010 is not very potent *in vitro*, with an IC_{50} of 0.45 μg/mL against colon 38, which is about 100-fold less active than combretastatin A4. In animal tests, however, it demonstrates good efficacy against rodent solid tumors and a wide range of human xenografts (74). Against colon 38, its T/C was significant at 2% in mice dosed orally at 100 mg/kg (71). E7010 is also effective against vincristine-, cisplatin-, and 5-fluorouracil-resistant cell lines *in vivo*.



35 **36** **37** **38**

A summary of the Phase I study of E7010 has been reported (75). The maximum allowable doses were determined to be 480 and 240 mg/m^2 for patients

receiving a single dose and 5 day repeated doses, respectively. Peripheral neurotoxicity was the major adverse effect observed, and seemed to be similar to that caused by vincristine and paclitaxel. Other adverse effects such as hematological and gastrointestinal toxicities were relatively mild and were dose dependent. E7010 demonstrated favorable absorption and elimination profiles in humans. The oral bioavailability of E7010 was at least 80% following single dose administrations, with half-lives ranging from 4.4 to 16.6 h. Total drug recovery in urine 72 hours after administration was 77.8%. The efficacy of E7010 was disappointing, and the reduction in tumor sizes were observed for only two out of 16 patients with uterine sarcoma or pulmonary adenocarcinoma after a single oral dose.

Compound **36** (ER-34410), bearing a dibenzodiazopine group, was the second antimitotic compound reported in this series (76). It is 2- to 3-fold more potent than E7010 against various human tumor cell lines *in vitro*. In an animal test against M5076 ovarian sarcoma, ER-34410 produced a T/C of 39% on day 21 following intravenous dosing at 50 mg/kg, which was comparable to that of E7010 given orally at 400 mg/kg.

Replacing the phenylamino group of E7010 with a pyridylvinyl group yielded a series of potent antimitotic agents represented by **37**. Compound **37** is at least 145 times more potent *in vitro* than E7010 against colon 38 and is efficacious in the mouse model against colon 26, achieving T/C of 4.7% at 50 mg/kg given orally (77).

Modifications on the structure of E7010 led to the discovery of the latest reported compound in this series: the *N*-(7-indolyl)sulfonamide **38** (ER-35744, E7070) (78). Subsequent studies found that E7070 possessed a different mechanism of action from E7010 (79). It is thought to affect the progression of the cell cycle in the G1 phase with inhibition of expression of cyclin E and phosphorylation of cdk2. Structurally, the 7-indolyl structure motif was found to be essential, and substitution by halogen or cyano groups at the C-3 position of the indole moiety sharply increases activities (80). In animal tumor regression trials, E7070 suppressed tumor growth and reduced tumor volumes of various human colon and lung cancers, and impressively, it completely eradicated HCT-116 colon and LX-1 lung cancers following intravenous administration of 25 mg/kg of the drug once a day for 8 days (81-82). E7070 was reported to be in phase I clinical trials (83).

Summary - The clinical and commercial success of taxanes has stimulated a world-wide search for antimitotic agents that possess superior profiles. The epothilones and their analogs provide us with a new frontier in search of taxane-like compounds that are active against MDR tumors. Among the promising compounds currently in clinical trials which interact with tubulin at sites different from those of taxanes are cemadotin, cryptophycin 52 and the CA-4 disodium phosphate (**19**). Recently, considerable efforts have focused on identifying new colchicine-site binders. These newer compounds demonstrate promising activity *versus* MDR phenotypes. The relatively simple structures of CSBs provide the added advantage of being orally active (84).

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Chapter 15. DNA Vaccines Coming of Age

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Introduction. State of the Art - Enormous progress has been made since the initial reports that transfection of cells *in situ* with bacterially-derived plasmid DNA could lead to *in vivo* expression of an encoded antigen, the induction of humoral and cellular immunologic responses, and protection from virus challenge in small animal models (1-5). In 1998, results from at least three independent early phase clinical trials of first generation DNA vaccines have been published that indicate that these vaccines are safe and immunogenic (albeit weakly) in humans (6-8; see below).

This progress to the clinic comes on the heels of numerous proof-of-principle studies that have demonstrated the ability of DNA vaccines to induce protective immune responses against infectious agents including viral, bacterial, and parasitic pathogens, as well as against various tumors (4, 9). However, the ultimate challenge for this technology remains – will DNA vaccines induce adequately potent, long-lasting, and protective immune responses against the most deadly infectious agents of the modern world, pathogens such as HIV, malaria, and tuberculosis.

This review describes the latest findings with regard to the basic immunology of nucleic acid (or "DNA") vaccines and will address the current approaches being evaluated to improve the potency and protective efficacy of immune responses after DNA vaccination. Approaches under evaluation include: increasing antigen expression and delivery, targeting of DNA or antigen to specific cellular compartments or cell types (e.g. antigen-presenting cells [APC]), co-delivery of chemical or genetic adjuvants to enhance antigen-specific immunity, use of vectored nucleic acid delivery systems such as attenuated bacterial strains or propagation-defective alphavirus replicons, and enhancement of DNA-primed immune responses through the use of mixed modality (prime-boost) vaccine regimens. These are discussed in greater detail below.

Basic Immunology of DNA Immunization - Like other vaccine approaches, DNA immunizations elicit antibodies and CD4+ T-cell responses, but their primary strength is their potential to induce potent antigen-specific class I major histocompatibility (MHC)-restricted cytolytic CD8+ T-lymphocyte responses (CTL). These latter responses are believed to be important for the control of intracellular pathogens and tumors. In addition, viral antigens (such as HIV Envelope proteins) encoded by DNA vaccines can be expressed *in vivo* in their native conformations which may result in more effective priming for the induction of relevant neutralizing antibody responses. Other vaccine strategies being evaluated to induce both CTL and antibody responses include infectious viral or bacterial vectored deliveries such as vaccinia, adenovirus, Salmonella, Listeria, and BCG, or non-infectious deliveries of subunit protein or other vaccines using saponin or MPL-based adjuvants or particulate deliveries. However, among these, DNA vaccination holds the most appeal and promise because of its reduced risks for infection and toxicity and for the relative ease of manipulation of DNA vectored vaccines.

The primary modes of DNA vaccine administration are intramuscular (IM) or intradermal (ID) by needle injection or by epidermal injection by particle-bombardment (gene-gun). The majority of plasmid DNA appears to be taken up and expressed by transfected muscles cells (for IM immunizations) and keratinocytes (for

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ID or gene-gun) at the sites of injection. However, since these cells are not professional antigen-presenting cells, it was suggested early on that these cells were not solely responsible for the induction of the observed immune responses. This presented researchers with the following paradox that since has been at least partially resolved: DNA vaccination was believed to potentially induce MHC-restricted CD8+ CTL responses by virtue of the intracellular delivery of antigen akin to the cytoplasmic delivery of antigens during viral infection, but the primary cells transfected by DNA immunization were not professional APCs and did not appear to have the requisite co-stimulatory molecules on their cell surfaces. Furthermore, while antigen expression in tissues at the site of injection could be long-lived, the majority of the injected DNA was rapidly degraded and believed to be unavailable for further transfection of other cells, such as migratory APC (2,5,9).

A clearer picture of the mechanisms of antigen presentation involved in DNA vaccination has since emerged. For IM DNA immunization, two groups used adoptive transfer of cell subsets in immunodeficient (irradiated or Severe Combined Immunodeficient [SCID]) mice to demonstrate that transfection and antigen expression in muscle cells was most likely sufficient for the induction of MHC-restricted CTL responses if bone marrow-derived dendritic cells of the appropriate MHC 1 type were available to present the antigenic epitope (10-12). Thus, while direct transfection of professional APC was not required for the induction of CTL responses during IM immunization, the presence of these cells in the immunized animals was found to be absolutely required. Two mechanisms to explain these observations have been suggested. In the first and more likely case, a transfer of antigen to professional APC referred to as "cross-priming" could be operating during DNA immunization by the IM route. Thus, even if the expression of plasmid encoded antigens were restricted to myocytes at the site of injection, these antigens could access an alternative pathway for class 1 MHC processing of the antigen that did not rely upon cytoplasmic expression in the APC itself. The existence of this alternative pathway had been previously demonstrated for presentation of tumor antigens, and has recently been shown to be active during the induction of CTL responses against viruses that do not directly infect APC (13, 14). Another explanation was that APC were indeed being directly transfected at an undetectable level during IM DNA immunization and these few transfected cells were responsible for the induction of MHC-restricted CTL. The relative contributions of these two mechanisms for the induction of CTL responses following IM DNA immunization are yet unresolved.

While myocytes are the only cells with detectable levels of antigen expression after IM immunization with naked DNA, intradermal administration of DNA results in expression in a variety of cell-types including some with dendritic cell morphology believed to be Langerhan's cells (LC) (5). LC are the professional APCs of the skin and when loaded with antigen from the periphery, they migrate to the regional lymph nodes where they differentiate to express co-stimulatory molecules and become potent activators of naïve T-lymphocytes. The initial observations for DNA vaccination were made in mice that received ID immunizations by needle injection, and have since been confirmed for ID immunization using particle-bombardment of the skin with the gene-gun (15). In these latter experiments, the skin cells of mice were painted with rhodamine and followed after DNA immunization. Rhodamine labeled (skin-derived) dendritic cells (LC) containing the gene gun-administered gold particles were found to localize in the draining lymph nodes where they expressed their DNA-encoded reporter gene, green fluorescent protein (GFP). Other studies have demonstrated a critical role for the presence of rare antigen-expressing dendritic cells in the lymph nodes draining the site of DNA delivery for the induction antigen-specific CTL responses, providing further evidence for the importance of directly transfected LC during gene gun immunization (16). More recently, the

contribution of cells at the site of ID DNA immunization has been further clarified by experiments in which the site of injection was excised and transplanted at various times following immunization with the gene gun (17). These studies showed that primary B- and T- cell immunity was induced by cells (most likely, skin-derived APC) that migrated from the site of immunization within a short time, but that nonmigratory transfected cells contributed to the magnitude of the primary response.

These findings with regard to the possible existence of distinct modes of antigen presentation after IM and ID DNA immunizations may have major implications for the design of vaccines. When selecting an immunogen, special consideration may have to be given to the route of administration and the cellular localization of the antigen in relation to the desired immune responses.

Adjuvants and Deliveries to Enhance Immune Responses - An immunologic adjuvant acts to accelerate, prolong, or enhance antigen-specific immune responses when combined with vaccine antigen(s). Three mechanisms have been proposed to explain how adjuvants serve to enhance immune responses to DNA immunization: 1) through recruitment of lymphoid cells including APC (macrophages or dendritic cells) to the site of DNA administration; 2) by the induction (or provision of) cytokines which can activate naïve lymphoid cells; or 3) by facilitation of DNA entry/transfection of the host cell(s) (18). An important safety consideration for DNA vaccines is that an adjuvant must serve to increase immune responses to encoded antigens without increasing responses to the DNA itself. Several chemical and genetic adjuvant strategies have been tested for their abilities to enhance humoral and cellular responses by DNA vaccination and these have been recently reviewed (18). Some of these strategies have been used effectively for both parenteral and mucosal delivery of DNA and have been shown to induce mucosal as well as systemic immune responses (18, 19). Since the majority of pathogens gain access to the host through mucosal surfaces, induction of immune responses at these sites is desirable. Nevertheless, most of the adjuvant and delivery strategies evaluated for systemic and mucosal delivery of DNA have been tested only in small rodents, and for the most part, have yet to be proven effective in primates.

The conventional adjuvant, aluminum phosphate (alum), was shown to enhance antibody responses to DNA vaccination in mice up to 100-fold and 5-10 fold in non-human primates through a mechanism that did not effect antigen expression *in situ* (20). Two other chemical adjuvants, widely in use to enhance immune responses to polypeptide-based vaccines, are monophosphoryl lipid A (MPL), a detoxified form of lipid A from the lipopolysaccharide (LPS) of a Salmonella strain, and QS-21, a highly purified triterpene glysoside saponin from the bark of the Quillaja saponaria tree. Both of these have been shown to enhance humoral and cellular immune responses to IM administered DNA vaccines (18, 21). Both adjuvants appeared to exert their effects through the induction of T-helper cell type 1 (TH1-type) cytokine responses, characterized by secretion of interleukin-2 (Il-2) and Interferon-gamma (IFN-γ), and by production of the antibodies of the IgG2a isotype (in mice). The induction of these TH1 responses generally correlated with the induction of cellular immune responses such as CTL. QS-21 was also shown to consistently enhance both mucosal and systemic immune responses through the intranasal (IN) route of DNA inoculation (21). QS-21 augmented both intestinal secretory IgA production and CTL activity in the spleen and mesenteric lymph nodes.

Cationic lipids and mannan-coated liposomes have been tested for their abilities to increase the transfection efficiency of entrapped DNA and thus, to indirectly enhance immune responses to the encoded antigen (18). (However, increased *in vitro* transfection efficiencies do not always predict increased transfection efficiency or immunogenicity *in vivo*.) One recent study employed intranasal delivery of the

reporter gene, firefly luciferase, complexed to the lipid DMRIE/DOPE to track gene expression *in vivo* and to monitor the induction of systemic and mucosal immune responses (22). IN immunization with the lipid:DNA complexes as compared to naked DNA alone resulted in enhanced dissemination and expression of the DNA throughout the mouse respiratory and gastrointestinal tracts, the draining lymph nodes, and spleens. More importantly, the enhanced gene expression observed with the IN administered lipid:DNA correlated with increased levels of circulating IgG and IgA, increased secretory IgA in the vaginal and rectal secretions, and the generation of antigen-specific CTL localized in the genital and cervical lymph nodes as well as the spleen. The use of mucosally administered lipid:DNA complexes could represent a promising approach for the induction of protective mucosal responses against sexually transmitted diseases. Accordingly, this approach is now being tested in an SIV-based mucosal challenge in non-human primate model (22).

The oral (or po) route of delivery represents another non-invasive and more convenient mode of DNA vaccine delivery. The gastrointestinal tract contains multiple immune inductive sites (lymph nodes and aggregations of lymphoid follicles call Peyer's patches) that comprise the gut-associated lymphoid tissue (GALT). The GALT is part of what is considered the common mucosal immune system and as such can serve as an immune inductive site for the priming of immune responses that can act at other mucosal surfaces such as those of the respiratory and urogenital tracts. Effective oral delivery of plasmid DNA requires that the DNA be protected against degradation from the gastric environment and that it be effectively delivered to immune inductive sites such as the Peyer's patches. One approach designed to meet these challenges has been to encapsulate the DNA in microparticles formulated with biodegradable poly (lactide co-glycolide) (PLG) (23). Orally administered PLG particles less than 10 micron in diameter have been shown to be phagocytosed by specialized cells in the gut (M cells) that overly the Peyer's patches. M cells transport particulates to APC in the Peyer's patches resulting in antigen-specific T- and B- cell stimulation and the induction of mucosal immune responses. Proof-of-concept experiments have shown that a single oral administration of plasmid DNA entrapped in PLG microparticles to mice can induce serum IgA, IgG, and IgM and high titers of IgA in fecal pellets (23). More recently, protective immunity against rotavirus challenge was induced in mice after a single oral immunization with a PLG-encapsulated DNA vaccine (24).

Co-delivery of genetic adjuvants in the form of plasmids expressing cytokines, chemokines, and co-stimulatory molecules has been widely explored as a means to enhance immune responses to DNA immunization (18, 25-29). The first experiments in this area were performed in the mouse rabies model and showed that co-injection of naked DNA encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) enhanced antibody and cellular immune responses against rabies glycoprotein antigen (25). These enhanced responses correlated with increased protection against rabies virus challenge in the vaccinated animals. The proposed mechanism to explain these results related to the ability of GM-CSF to induce differentiation of hematopoietic progenitors (such as monocytes or immature dendritic cells) into professional APC, thus increasing the efficiency of antigen presentation, and enhancing antigen-specific immunity.

More recent experiments in a herpes simplex type 2 mouse model showed that co-administration of TH1-type cytokines with a glycoprotein D DNA vaccine could reduce mortality and severity of disease following vaginal virus challenge while co-administration of TH2 cytokines (Il-4 or Il-10) actually increased the severity of disease (26). Of the TH1 cytokines tested (Il-2, Il-12, Il-15, Il-18), Il-12 co-delivery demonstrated the most dramatic vaccine effect which correlated with the highest ratio of anti-gD specific IgG2a to IgG1, indicative of enhanced cellular immunity.

Others have previously reported enhanced cell-mediated immune responses employing Il-12 plasmid co-administration by either IM or IN routes (27, 28). Chemokines, such as the murine beta-chemokine, TCA3, have been evaluated for their chemotactic activity towards inflammatory cells such as macrophages and thus, their potential abilities to recruit APC to the site of DNA injection. Increased accumulation of mononuclear cells in injected muscles as well as enhanced CTL responses have been reported (18). Co-delivery of plasmids encoding co-stimulatory molecules has been used in attempts to improve the antigen-presenting capabilities of myocytes (or other transfected cells) after DNA injection. Co-administration of B7-2 (but not B7-1) with plasmid DNA encoding an antigen was found to specifically enhance CTL responses through a mechanism that required B7 interaction with CD28 (on T-cells) as well as the presence of IFN-γ, indicating that it was mediated by active co-stimulation (29).

Finally, the presence of unmethylated bacterial "CpG" DNA sequence motifs (an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines) or "ISS" (immunostimulatory sequences) in plasmid DNA has been shown to enhance both antibody and CTL responses to DNA immunization (30, 31). These sequence motifs are underrepresented in vertebrate genomes and when administered in bacterial plasmid DNA vectored vaccines were reported to induce a T-cell independent, antigen non-specific B-cell activation that synergizes with antigen-specific B-cell activation to enhance Ig secretion (32). CpG's were also shown to activate monocytes/macrophages and dendritic cells to upregulate co-stimulatory molecules and to secrete or otherwise induce high levels of TH1 cytokines such as Il-12 and IFN-γ. Recently, additional sequence motifs were identified that appear to neutralize the immunostimulatory effects of the CpG (33). Thus, the presence of both the stimulatory and neutralizing sequence motifs may have to be considered in the design of optimal DNA vaccine constructs. Lastly, CpG oligonucleotides were shown to enhance and modulate the immune responses when used in combination with protein antigens alone or formulated with adjuvant (34). The addition of CpG DNA to a hepatitis B surface protein vaccine in the TH2 adjuvant, alum, led to both an increase in antibody titers and a shift from a TH2 to a TH1-type response characterized by the appearance of antigen-specific CTL activity and increased IgG2a production in mice. These results highlight the potential usefulness of approaches that utilize co-administration of protein- and DNA-based vaccines.

Vectored Delivery of Nucleic Acid - Nucleic acids have been effectively delivered to the immunized host by bacterial or propagation-incompetent viral vectors. For bacterial delivery, attenuated strains of Listeria, Salmonella, Shigella, and E. coli have been employed to carry plasmids bearing cloned DNA into host cells, and have demonstrated the feasibility of this approach for DNA immunization (35-39). A major advantage of enteric bacterial-mediated DNA delivery lies in the ability of these organisms to invade and carry plasmid DNA into gut-associated lymphoid cells (APC) by a pathway similar to that described above for oral delivery of PLG microparticles. Plasmid DNA released by the intracellular bacteria can be expressed by the host cell leading to both systemic and mucosal immune responses. Attenuated Salmonella strains have been widely used as live vaccines in humans and animals and would appear to be ideal for candidates for DNA vaccine delivery. However, they were believed to be of limited use as carriers of plasmid DNA for induction of CTL responses because, in contrast to Shigella and E. coli strains that escape the phagolysosome to gain access to the cytoplasm, these strains were believed to be retained within this compartment. Nevertheless, recent studies have provided evidence that oral immunization with Salmonella vectored plasmid DNA can lead to host cell expression of the encoded antigens and the induction of MHC-restricted CTL responses (36, 37). The future challenge for

bacterial delivery may be in the derivation of strains with the necessary balance of attenuation and invasiveness to achieve adequate safety and vaccine potency.

Of the viral vectored deliveries for nucleic acid vaccines, recombinant alphaviruses presently are receiving increased attention. The broad host range and high levels of expression of proteins encoded by these viruses have made them attractive for vector development. Alphaviruses contain single-stranded positive RNA genomes and replicate in the cytoplasm of the infected host cells, without the need for a DNA intermediate. This feature may provide an important safety advantage over naked DNA vaccines with regard to concerns about the possibility of DNA integration into the host genome and the generation of anti-DNA antibodies. The first alphavirus expression vectors were based on the Sindbis virus, followed by analogous vectors based on other similar virus strains including Semliki Forest virus (SFV) and Venezuelan equine encephalitis virus (VEE) (40-42). The vectors derived from these alphaviruses consist of viral cis regulatory elements, four nonstructural regulatory genes, and the viral subgenomic promoter that can drive high level expression of the antigen of interest. No alphaviral structural protein genes are encoded by alphavirus vectors, and virus particle production is entirely dependent on the co-transfection of cells with the structural genes encoded by separate plasmids, or on transfection of stable packaging cell lines. The alphavirus vectored vaccines can be delivered as naked RNA or packaged as virus particles, or more recently, as a DNA-based vectors (cDNA copies of the RNA replicon driven by a RNA polymerase II promoter) (43).

Two independent studies have demonstrated improved vaccine efficacy of the DNA-based alphavirus vectors (Sindbis and SFV-derived) over naked plasmid DNA (44, 45). In these experiments, substantially smaller doses of injected alphavirus vector DNA as compared to plasmid DNA were required to induce antibody and CTL responses as well as protection in viral challenge experiments conducted in mice. The increased efficacy of the alphavirus-vectored vaccines was proposed to be due to higher expression of the antigen and/or an adjuvant effect of the virus-derived double-stranded RNA replication intermediates which could induce the production of interferons and upregulation of MHC class I molecules (44). Furthermore, host cells containing alphaviruses or vectors derived from them die shortly after infection and undergo apoptosis. This process in itself may lead to increases in the efficiency of antigen uptake by APC (e.g. by cross-priming for CTL induction) and enhanced antigen-specific immune responses. Lastly, important advances in the development of stable packaging cell lines for the production of high titer alphavirus particle preparations further indicates the feasibility of alphavirus vector systems for vaccine applications (46).

Prime-Boost Immunization Strategies - One of the most promising strategies to enhance the immunogenicity and the protective efficacy of DNA vaccines is through the use of mixed modality (or "prime-boost") immunization strategies (47). This type of combination vaccine was pioneered employing recombinant poxvirus-vectored vaccines encoding HIV antigens (48). Enhanced neutralizing antibody responses and protection against immunodeficiency virus (SIV) challenges were observed in primates that received recombinant live vaccinia virus expressing Envelope (Env) proteins followed by booster immunizations with recombinant subunit Env proteins (48). In addition, prime-boost regimens employing recombinant canarypox vectors expressing multiple HIV antigens in combination with adjuvanted Env proteins are now in advanced clinical trials (49). So far, these vaccines raise modest CTL responses and high titer neutralizing antibody responses. Hence the need for improved strategies such as DNA vaccination for the induction of more robust cellular immune responses.

Similar prime-boost regimens with HIV Env-based DNA vaccines showed that neutralizing antibodies induced after primary immunization with plasmid DNA delivered by the IM or by the ID (gene-gun) routes can be enhanced 1-2 logs by subsequent boosting with Env protein preparations (50, 51). Moreover, primates that received IM DNA immunizations with plasmids encoding HIV Env followed by boosting with Env protein showed potent CTL, high titer neutralizing antibody responses, and protection against immunodeficiency virus challenge (52). In contrast, primates immunized with SIV or HIV-based DNA vaccines delivered by the gene gun either with or without protein boosting were not protected (53-55).

The DNA plus protein mixed modality strategy is particularly appealing for vaccination against HIV because of the potential to induce potent, broad, and long-lasting humoral and cellular responses (47). It may be possible to employ a multigenic (several genes represented) and multivalent (different HIV strains or subtypes included) DNA vaccine component in combination with a limited representation of conserved Env protein structures for the induction of broad CTL and neutralizing antibody responses against diverse HIV strains.

Other mixed modality vaccine strategies that are being explored focus primarily on the induction of potent CTL responses. Noteworthy among these are DNA plus recombinant Modified Vaccinia Ankara (MVA) (56), and DNA plus recombinant Fowlpox Virus (57). The DNA prime/ MVA boost regimen was shown to be effective for the induction of protective MHC-restricted CD8+ CTL in a mouse malaria model while single modality vaccines were ineffective (56). More recently, resistance to intravenous challenge with HIV-1 was demonstrated in macaques (Macaca nemestrina) that received two gene-gun DNA immunizations with plasmid DNA encoding HIV Env and Gag followed by three booster immunizations with recombinant Fowlpox vectors encoding Env, Gag, and Pol (57). Following the Fowlpox booster immunizations, all vaccinated animals showed substantial HIV-specific cellular (CTL and lymphoproliferative responses) and humoral responses and all were protected from challenge with the homologous HIV-1 train. However, this study must be interpreted with caution in that no other vaccine regimens were tested or compared directly in the primate model employed.

Progress to the Clinic - Results of several human clinical trials of DNA vaccines against infectious diseases have been reported in the past year (6-8). Two of these trials evaluated immunizations with HIV antigens in asymptomatic HIV-infected subjects and a third was performed with malarial antigens in naïve subjects (6-8). In the first, conducted in the US, individuals received a dose-escalation of intramuscularly administered plasmid DNA encoding the HIV Envelope glycoprotein (gp160) (6). Individuals receiving the highest doses of DNA (100 ug or 300 ug) exhibited (anamnestic) increases in anti-Env antibodies. Enhancement of T-cell immune responses such as antigen-specific lymphoproliferation and major histocompatibility (MHC) class 1-restricted cytotoxic T-cell activity (CTL; see below) was difficult to interpret due to high pre-existing levels of these activities in HIV-infected people. In the second, a cohort of asymptomatic HIV-infected patients in Sweden received intramuscular injections of plasmids encoding the HIV regulatory genes, *nef, tat,* or *rev* (7). This group was pre-selected on the basis of no or low pre-existing responses to these antigens, and after immunization, eight of nine patients showed evidence of increased CTL and lymphoproliferative responses. The third trial was a randomized dose escalation employing IM immunization of 20 uninfected subjects with plasmid DNA encoding the *P. falciparum* circumsporozoite (liver-stage) protein (8). Nine of these individuals exhibited MHC-restricted CTL responses, and failure to respond was attributable to lower dosage or absence of the appropriate histocompatibility alleles (HLA) for the CTL epitopes tested.

Combined, these early clinical studies clearly demonstrate the safety and immunogenicity of DNA vaccination in humans and suggest the feasibility of this approach for the development of effective vaccines. Future clinical evaluations of several of the new technologies described here promise to improve the immunogenicity and protective efficacy of the next generation of DNA vaccines.

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Chapter 16. Development of Anti-Malarial Inhibitors of Hemoglobinases

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Introduction - Malaria is the fourth largest infectious disease in the world, behind diarrhoeal diseases, tuberculosis and measles (1). Caused by protozoan parasites of the genus *Plasmodium*, the disease is transmitted to the human host by the bite of the female *Anopheles* mosquito. There are estimated to be between 300 to 500 million clinical cases of malaria per year, with an estimated mortality of 1.5 to 2.7 million per year. The majority of these deaths are in children under the age of five. In 1994, it was estimated that nearly 2.3 billion people lived in areas at risk for malarial transmission, which is nearly 41% of the planet's population (2). Although worldwide in distribution, 90% of cases are reported from tropical Africa.

There are several factors which have caused increased concern regarding the transmission and spread of malaria. One factor is the rapid and unprecedented movement of naïve human populations into endemic areas. For the first time in human history, vast human migrations, both temporary and permanent are taking place. In the early 1990's, it was estimated that over 500 million people crossed international boundaries every year (3). These migrations are being fueled by rapid transportation and an expanding human population. This expanding population is also increasingly encroaching upon formerly rural endemic areas (4). Secondly, environmental degradation is of concern. Global warming has the potential for both disrupting current patterns of transmission and in potentially expanding the range of the *Anopheles* mosquito (5). The driving force for concern, however, is the increasing resistance to current anti-malarial chemotherapy (6). New drugs for the treatment of malaria are needed. Compounds used as mainstays of treatment are rapidly losing their efficacy when used alone or as combination agents against the major causative agent of malaria, *P. falciparum*. This is especially true of chloroquine , the first line therapy for the last forty years,. In 1994, only Central America and Egypt had not recorded evidence of chloroquine resistant *P. falciparum*. Several new classes of anti-malarial agents have been developed in recent years, but most suffer from significant drawbacks (7). A recent review in this journal has detailed the current state of anti-malarial chemotherapy. One new and promising approach in anti-malarial therapy is the use of protease inhibitors for interfering with the organism's ability to degrade and utilize hemoglobin as a source of nutrients. This review will focus upon recent advances in the development of protease inhibitors as potential therapeutics for the treatment of malaria.

BIOLOGY OF HEMOGLOBIN DEGRADATION

Life Cycle and Hemoglobin Utilization - Initiation of infection with *P. falciparum* begins with the bite of an infected female *Anopheles* mosquito, injecting the sporozoite form of the parasite into the bloodstream of the host. The sporozoites then enter the liver through invasion of the hepatic parenchymal cells and there they mature into the merozoite and are released from the liver back into the bloodstream. Merozoites invade erythrocytes through a series of complex interactions, eventually establishing themselves inside the erythrocyte, sequestered from immune surveillance. Merozoites develop further through a series of morphologically differentiated forms within the erythrocyte, asexually reproduce to form schizonts, and eventually yield up to 24 new merozoites. These merozoites rupture the erythrocyte and quickly invade a new erythrocyte.

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During schizogony, the trophozoite intermediate form develops a large acidic food vacuole. Within the food vacuole, erythrocyte hemoglobin is degraded. The acidic food vacuole has been shown to contain proteolytic enzymes, three of which have been specifically characterized. The plasmepsins I and II are aspartyl proteases. They have acidic pH optima and are homologous to other known aspartyl proteases, most notably lysosomal cathepsin D. In addition to these aspartyl proteases, a cysteine protease, falcipain, has been identified as playing a role in hemoglobin degradation.

One by-product of the degradation of hemoglobin is the accumulation of hematin into the reddish-brown hemazoin pigment. Heme is toxic to the parasite, and its accumulation into the hemazoin polymer sequesters the toxic heme. It is believed that the weak base quinoline anti-malarials exert their toxic effect on the parasite by interfering with the heme sequestration process (8).

Essential Nature of Hemoglobin Degradation - There are several lines of evidence, from *Plasmodium* strains other than *P. falciparum*, suggesting that degradation of hemoglobin is an essential function in malarial parasite growth and survival. The malarial parasite has a limited capability to synthesize amino acids, therefore it must obtain amino acids from its intra-erythrocytic environment. The free amino acid concentration in erythrocytes is limited and not sufficient to support parasite growth and differentiation. During the parasite life cycle within the erythrocyte, up to 80% of the hemoglobin is degraded to free amino acids. This is evidenced by the disappearance of hemoglobin within infected erythrocytes. There is a concomitant increase in the concentration and composition of free amino acids within the infected erythrocyte, compared to uninfected erythrocytes, which is similar to the amino acid composition of hemoglobin. In addition, using radiolabeled hemoglobin, the uptake of radiolabeled amino acids into parasite proteins within the infected erythrocyte has been demonstrated (9).

Aspartyl and Cysteine Proteases Are Responsible for Hemoglobin Degradation- Early evidence using *P. falciparum* trophozoites suggested that both aspartyl and cysteine proteases are involved in hemoglobin degradation. Incubation of *P. falciparum* trophozoites with the protease inhibitor leupeptin led to the accumulation of undegraded hemoglobin within the food vacuole. Leupeptin is both an aspartyl and cysteine protease inhibitor. Using a more specific cysteine protease inhibitor, E-64, similar results were obtained. Other classes of protease inhibitors were found not to effect hemoglobin degradation. In isolated food vacuoles, pepstatin, a specific aspartyl protease inhibitor was found to inhibit hemoglobin degradation to a greater extent than E-64 (9). In addition, when organisms are grown in culture depleted in exogenous amino acids, they become dependent solely upon degradation of hemoglobin and therefore are more sensitive to protease inhibitors (10).

The precise mechanism of hemoglobin degradation remains unclear. The initial cleavage of native hemoglobin has been attributed mainly to the cysteine protease, falcipain. This is based upon inhibition of hemoglobin degradation and parasite growth and development by specific cysteine protease inhibitors (7). Other evidence suggests that plasmepsin I is also capable of cleaving native hemoglobin, but only under the non-reducing conditions likely to be present in the food vacuole. Under similar conditions, falcipain was shown not to cleave native hemoglobin (7). Although the precise mechanisms of hemoglobin degradation remain unclear, hemoglobin degradation in malarial parasites remains an area of active research and development. All three proteases, so far identified as playing a role in hemoglobin degradation, plasmepsin I and II and falcipain, have been selected as primary targets for the development of inhibitors.

PLASMEPSINS

Plasmepsin Biosynthesis and Maturation- The genes for plasmepsins I and II have been cloned and are predicted to encode proteins of approximately 51 kDa. The two plasmepsins share significant homology, being 73% identical. They are 35% homologous to cathepsin D. Plasmepsin I and II are similar to other aspartyl proteases as they are secreted proteins and expressed as pre-pro-proteins. The pro-sequence of the plasmepsins is longer than with traditional aspartyl proteases. In addition, plasmepsins have a hydrophobic predicted membrane spanning region in the amino terminal of the pro-protein. This region is believed to be a signal anchor sequence (11). Both plasmepsins I and II are capable of making the initial cleavage of the alpha chain of hemoglobin at the Phe-33/Leu-34 peptide bond.

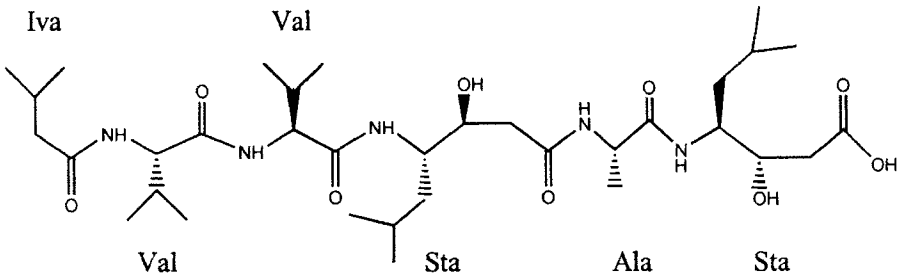
Processing of pro-proteins in most aspartyl proteases is by autoactivation at low pH. This was believed to be the mechanism for plasmepsins until recently. The processing of the proplasmepsin I has been shown to be inhibited by tripeptide aldehydes ALLN and ALLM. These compounds inhibit cysteine proteases and it is proposed that proplesmepsin I processing is mediated by another enzyme and not by autoactivation. The tripeptide aldehydes did not inhibit proplasmepsin II maturation. The putative cysteine protease involved in proplasmepsin I maturation is not a traditional cysteine protease, as this activity could not be inhibited by other cysteine protease inhibitors, such as E-64. Falcipain was thus ruled out as a candidate for this activity (11).

Expression of Plasmepsins I and II - Plasmepsins are expressed *in vivo* as pre-pro-proteins. Expression of these enzymes in an active form was necessary to use these targets for screening and drug development studies. Expression of the full length pre-pro-proteins resulted in low yields of enzyme (12). To overcome this limitation, truncated precursor proteins of both plasmepsin I and II were expressed, isolated as inclusion bodies, solubilized in urea and refolded to yield pro-enzymes. For plasmepsin II, this worked well and the pro-protein was able to undergo autoactivation at low pH to yield active plasmepsin II. Proplasmepsin I was unable to undergo autoactivation to produce active enzyme. This problem with proplasmepsin I was solved by altering the sequence of the pro-protein to exchange a lysine with a valine residue. Based upon observations made with the autocatalyticaly active proplasmepsin II, the positively charged Lys-110 was predicted to be in the S2 pocket of the active site in proplasmepsin I . This is known to be unfavorable for aspartyl proteases. Alteration to Val-110 provided autocatalytically active plasmepsin I (12, 13). These observations on recombinant plasmepsin I autoactivation are interesting in light of the recent work, described above, indicating that *in vivo* proplasmepsin I may be activated by another enzyme (11).

Structure Based Design of Peptidomimetic Inhibitors of Plasmepsins - Plasmepsins are 30% homologous to the five known human aspartyl proteases. Using recombinant plasmepsin II, the structure of this enzyme was compared with the known structures of cathepsin D and renin. Plasmepsin II structure showed greater similarity to cathepsin D than to renin, and all three were noted to be very conserved in active site loop regions. Cathepsin D and plasmepsin were also compared in pepstatin A complexes. The bound conformation of pepstatin A displayed a larger solvent accessible area in plasmepsin II than in cathepsin D. The "flap" region of plasmepsin II was also bulkier and more hydrophobic than in either cathepsin D or renin, and thus may lend different characteristics to inhibitor or substrate binding (10).

The structural determination of the pepstatin A molecule in plasmepsin II has provided insight into the development of peptidomimetic inhibitors based upon the

statine core of pepstatin A (**1**). As part of a development effort for inhibitors to cathepsin D, inhibitors with potency against plasmepsins were also examined (14). Table 1 illustrates a series of peptidomimetics based upon a phenylalanine statine core. Generally, peptide inhibitors are not therapeutically useful due to their metabolic instability and poor physical properties. One approach to overcome these deficits is to conformationally restrict the peptides by creation of cyclized molecules, such as **2b**. Unfortunately, in most cases, information regarding the specificity of the inhibitors for the plasmepsins versus cathepsin D was not described. For those cases in which comparisons are available, specificity was limited. In addition, no supporting *in vitro* or *in vivo* efficacy data was available.

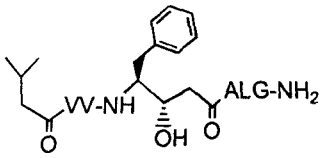
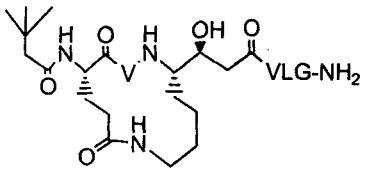
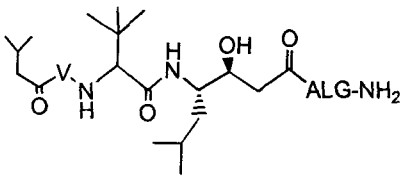
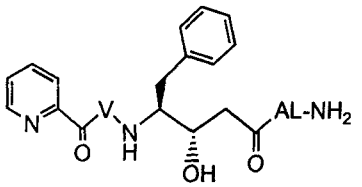


1

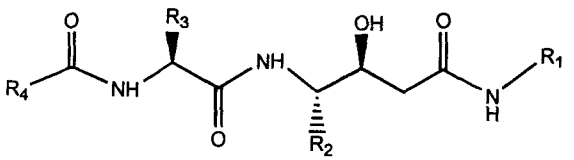
Combinatorial Library Synthesis for Inhibitors of Plasmepsins - Another group has reported the use of the core statine peptide (**3**) as a scaffold for building peptide inhibitor libraries of aspartyl proteases (15).

These libraries were screened against plasmepsin II and cathepsin D using fluorescence resonance energy transfer (FRET) assays in a high throughput mode. Libraries were synthesized using a split pool strategy on encoded *solid phase* supports (15). A comparison of 2,815 individual inhibitory compounds was performed by determining the per cent inhibition of activity for each compound for each enzyme, plasmepsin II and cathepsin D, and plotting the results for one enzyme versus the other. Compounds with a high specificity for plasmepsin II were identified. For compounds with preference for plasmepsin II, only Ile and Val side chains were observed in the R_3 position. Interestingly, the isobutyl group of leucine, another branched alkyl chain, was not found in the R_3 position of plasmepsin inhibitors. Hydrophobic groups in the R_1 and R_2 positions were necessary for inhibition, but did not allow for any discrimination in inhibition between enzyme targets. The R_4 position could be substituted with a number of substituents, indicating a high level of structural diversity is tolerated in this position for inhibition of plasmepsin II. In cathepsin D, the R_4 position was more limited in allowable structural diversity. The candidate inhibitors were re-synthesized and their inhibitory activity confirmed. Several potent inhibitors of plasmepsin II were identified. For example, PS273800 (**4**) had a K_i = 50 nM for plasmepsin II and a selectivity of greater than 6-fold when compared to inhibition of cathepsin D. Several compounds resulting from this library were tested in cell culture against *Plasmodium falciparum* and had IC_{50} values in the 10 μM range.

TABLE 1

Compound Structure[1]		Inhibition Constants (nM)		
		Plasmepsin I	Plasmepsin II	Cathepsin D
	**2a**	1.2	0.1	N.D.
	**2b**	22	0.2	0.26
	**2c**	7	3	N.D.
	**2d**	16	0.9	21

[1] Structures from ref 14. Single letter amino acid code; V= valine, A= alanine, L = leucine, G = glycine.



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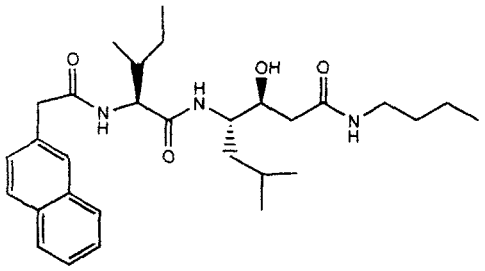
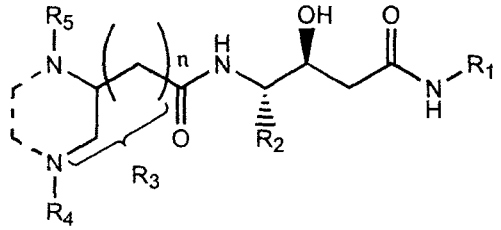


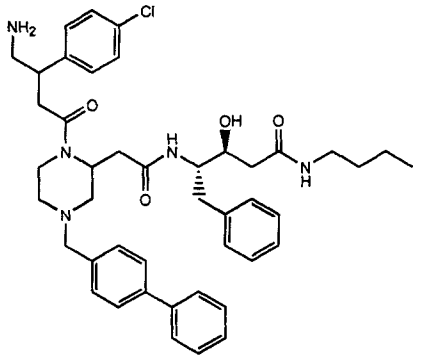
4

A second library was prepared using a scaffold determined by a docking exercise using the X-ray structure of the cathepsin D - pepstatin A complex (16). This exercise suggested that a functionalized piperazine acetic acid scaffold (5) was sterically acceptable in the active site of cathepsin D. This new scaffold allowed for exploration of the conformational space in the S_3-S_4 subsites which were not examined in the first library prepared by the group as described above.



5

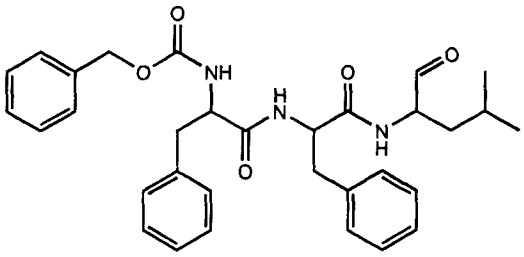
An analysis of the positional preference for inhibitors in the library was prepared by comparison of the frequency of appearance of any given substituent for inhibition of either plasmepsin II or cathepsin D. This comparison revealed that both enzymes favored alkyl amides in the R_1 position and benzyl in the R_2 position. There was a clear preference for two piperazine containing groups over proline in the R_3 position for inhibition of cathepsin D, whereas inhibition of plasmepsin II was favored with only one of the same two piperazine substituents. For the piperazine subsitituents, however, the stereochemistry of the active compounds was not known, as starting materials were racemic mixtures. Analysis of the R_4 and R_5 positions did not reveal any differential trends, both enzymes favoring large hydrophobic arylalkyl groups. There were, however, specific instances of preferred substituents for plasmespin II inhibitory activity in the R_5 position. Compounds were re-synthesized, purified and re-tested for their inhibition of both enzymes. One compound, PS172564 (6), showed inhibition of plasmepsin II with a K_i = 490 nM and a 90-fold selectivity over inhibition of cathepsin D. No biological data on inhibition of *Plasmodium falciparum* growth or viability was reported.



6

Screening of Aspartyl Protease Inhibitors for Inhibition of Parasite Growth - The aspartyl protease cathepsin D has been reported to play a role in processing of beta amyloid precursor protein in the development of Alzheimer's disease (17). This has stimulated interest in aspartyl protease inhibitors in industry. Libraries of aspartyl protease inhibitors are now available at various pharmaceutical companies. One group has taken the direct approach. Using a medium throughput screen, several hundred aspartyl protease inhibitors were screened for inhibition of parasite growth in culture (12). Two compounds were identified which had IC_{50} values in the sub-micromolar range. The more potent of these, Ro 40-4388 (7), had activity against both a drug sensitive strain and a multi-drug resistant strain of *Plasmodium falciparum*. The IC_{50} for this compound is approximately 25-fold less potent than the IC_{50} for chloroquine against the drug sensitive strain.

Availability of active recombinant plasmepsin I and plasmepsin II allowed for activity analysis of these inhibitors against each of the plasmepsins. Plasmepsin I was 80-fold more sensitive to 7 than was plasmepsin II. In addition, using the structure of plasmepsin II, the structure of plasmepsin I was modeled, indicating that there could be significant differences in the S1 and S3 pockets of these enzymes that might account for the differential activities noted with these inhibitors. It is also interesting to note that 7, although peptidic in nature, deviates from the statine core used by others in development of aspartyl protease inhibitors against plasmepsin II. The activity of 7 with cathepsin D still shows a lack of selectivity, as the K_i for plasmepsin I is 4 nM and the K_i for cathepsin D is 70 nM. Thus, the selectivity of 7 is greater between the two plasmepsin species than it is for plasmepsin I and cathepsin D (18).

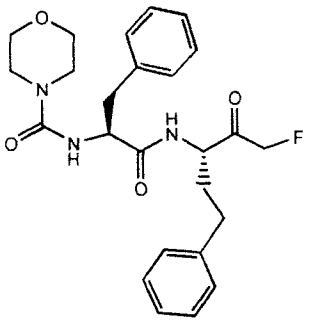


7

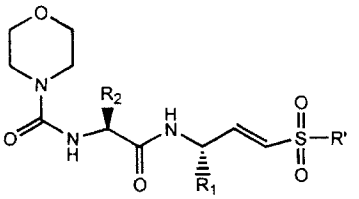
FALCIPAIN

Evidence for the Involvement of Falcipain in Hemoglobin Degradation - Falcipain is a cysteine protease with the biochemical characterisitics of food vacuole hemoglobin degrading enzyme. Cathepsin L, a mammalian homolgue, and falcipain are 37% identical (9). Falcipain has been shown to degrade both native and denatured hemoglobin *in vitro*, and has an acidic pH optimum characteristic for acidic organelle proteases. The substrate specificity and inhibitor sensitivity indicate that falcipain belongs to the papain family of cysteine proteases (7). It has been suggested that falcipain is expressed predominantly in the early ring and trophozoite forms of the intra-erythrocytic parasite (19). In contrast, plasmepsin I expression is detected in the ring stage, while plasmepsin II expression was found predominantly in the trophozoite stage (13). Although as described above for the plasmepsins, the precise ordered pathway for hemoglobin degradation remains unclear, there is evidence that blockage of cysteine protease activity by specific inhibitors blocks hemoglobin processing and degradation in cultured *P. falciparum* (7, 20).

Development of Inhibitors to Falcipain - Early work using specific fluoromethyl ketone peptide inhibitors of cysteine proteases suggested that potent inhibitors of falcipain could be useful as anti-malarial compounds. For example, **8a** had an IC_{50} for parasite growth *in vitro* of 0.03 μM . In an animal model of *P. vinckei* infection, this peptide cured 80% of murine malaria infections when dosed for 4 days (7). This experiment suggests that the falcipain and other proteases involved in hemoglobin degradation may prove to be useful targets for anti-malarial drug development. The toxicity of the fluoromethyl ketones in animal studies prevented further development of this class of compounds (19). Vinyl sulfones (**8b**), which have been shown to be inhibitors of cysteine proteases, have been utilized to develop similar peptide inhibitors to the fluoromethyl ketone series (21).

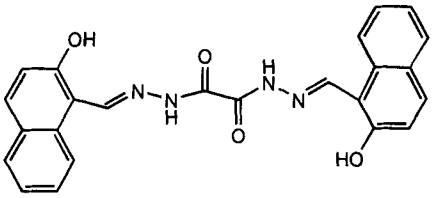


8a



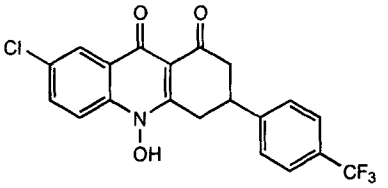
8b

More recent work has used a structure-based design approach. Construction of a homology-based structure of falcipain was performed using the known structures of papain and actinidin. Falcipain has 30% overall homology to these cysteine proteases, but in the active site region is approximately 60% homologous. Using the DOCK algorithm in combination with rigid body minimization algorithm, **9** scored highest for shape complementarity (22). This compound was tested and had an IC_{50} of 7 μM in a fluorescence activated cell sorting (FACS) assay for *Plasmodium* infected erythrocytes. Further medicinal chemistry studies using this lead compound resulted in a series of chalcones with sub-micromolar anti-malarial activity and some specificity over mammalian cysteine protease homologues.

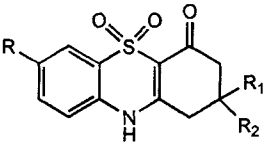


9

Acridinediones (10) have previously been shown to have anti-malarial activity, but their mechanism of action remains unknown. From the series of chalcones previously identified as inhibitors of falcipain, a conformationally restricted series was synthesized (23). This conformationally restricted series utilized a sulfur isostere of the acridinediones, the phenothiazines (11).

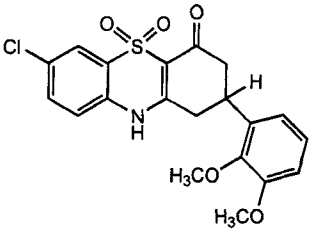


10



11

Ten compounds represented by structure 11 had IC_{50} values for falcipain inhibition in the 5 μM range. Using tritiated hypoxanthine uptake as a measure of parasite viability, eight of ten showed similar IC_{50} values. The two less potent compounds lacked an aromatic ring in the R_2 position. Although not as potent as the acridinediones, the phenothiazines have desirable solubility and toxicity properties and are used clinically. However, the mechanism of action of this series is still open to question. Compounds which lacked the aromatic ring in the R_2 position were still relatively potent inhibitors of falcipain and their anti-parasite activity may be due solely to this mechanism. Other compounds, including the lead compound (12) may act through inhibition of both falcipain (IC_{50} = 4μM) and by the mechanism, albeit unknown, of the acridinediones (23).



12

Conclusion- The degradation of hemoglobin has been shown to be a useful target for the development of anti-malarial compounds. Although no protease inhibitors have yet found clinical utility and efficacy data is scarce, hemoglobin degradation as an anti-malarial target is supported by the recent observation of synergy between aspartyl and cysteine protease inhibitors in prolonging survival in an infected mouse model (24). However, most of the compounds investigated are peptide inhibitors of the enzymes involved in hemoglobin degradation. Useful therapeutics will depend upon the development of specific, non-peptide inhibitors. The recent intense

industrial interest in proteases as targets for a number of therapeutic applications will add impetus to the development of potent and specific inhibitors of the malarial proteases for the treatment of *P. falciparum* infection.

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Chapter 17. New Antibacterials for Resistant Organisms

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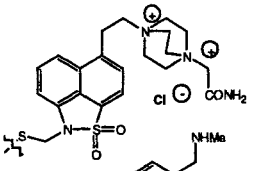
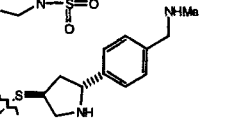
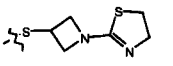
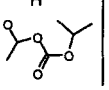
Introduction - The last decade of the 20th century has brought with it vancomycin-resistant *S. aureus* (1, 2). These glycopeptide intermediate resistant strains (GISA) may receive the most public attention of the resistant bacteria but they represent only the tip of the iceberg. Reviews this year have been written about resistance in general (3-6), about multidrug efflux in Gram-negative pathogens (7), about vancomycin resistance in the enterococci (8), and about β-lactam resistance in *S. pneumoniae* (9). There continues to be a constant call for new agents with new mechanisms of action (10,11). In the following review we will focus on new agents that have been reported in the past year. We will also provide an update on several compounds that have been reviewed in the past but for which there has been compelling new data in 1998.

β-Lactams - A new parenteral carbapenem, L-786,392, **1**, was reported to have excellent Gram-positive activity *in vitro* (Tables 1 and 2). The compound was superior to imipenem in *vivo*, including efficacy in a vancomycin resistant enterococcal (VRE) thigh model. This is believed to be due to lower plasma clearance and the resulting improved pharmacokinetics (12-14). The compound has reduced immunotoxicity in monkeys compared to earlier compounds in the series (15). A series of parenteral carbapenems, represented by J-111,225, **2**, with good activity against methicillin resistant *S. aureus* (MRSA) and anti pseudomonal activity but with diminished potency against *E. faecium* was presented (16). Pharmacokinetics and toxicity (17), *in vivo* efficacy (18), and β-lactamase inhibition of **2** were also revealed (19).

Table 1

	R^1	R^2
1	H	
2	H	
3	H	
4	$CH_2Ot\text{-}Bu$	
5	H	
6		

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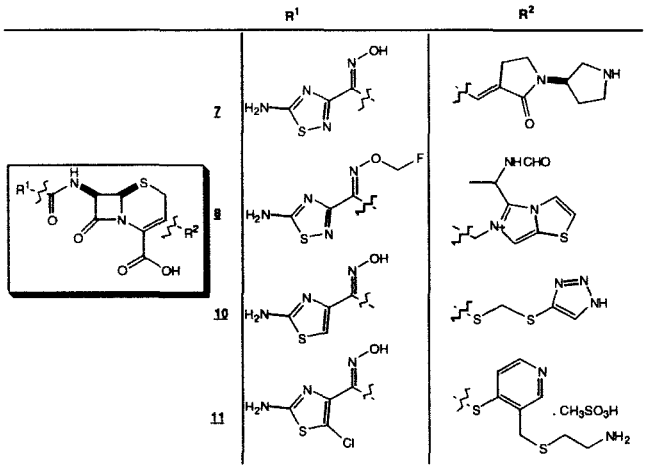
The active component, LJC 11,036, **3**, of oral carbapenem L-084, **4**, was reported to have potent broad spectrum activity against MRSA and PRSP and notably this compound had activity against GISA but not VRE (20, 21). Good efficacy of **4** in murine protection models against penicillin resistant *S. pneumoniae* (PRSP) was observed and efficacy similar to cefditoren pivoxil against *H. influenzae* was also demonstrated (22). The synthesis, *in vitro* and *in vivo* activity, pharmacokinetics and safety of KR-21056, **5**, the active component of oral prodrug, KR-21012, **6**, were described (23-26).

Ro 63-9141, **7**, which belongs to the pyrrolidinone-3-ylidinemethyl class of cephems demonstrated a potent *in vitro* spectrum of activity which included MRSA, MRSE, PRSP, and vancomycin-resistant *E. faecalis* and was less active against

Table 2

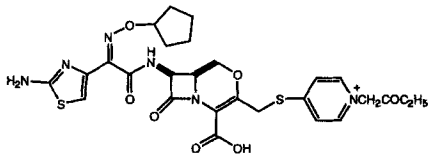


Organism	**1**	**2**	**3**	**7**	**9**
MRSA	4	4	6.25	4	6.25
MR-CNS	4	4	n/a	2	3
GISA	4	n/a	6.25	n/a	n/a
PRSP	0.5	0.25	0.05	2	0.05
E. faecalis	2	8	1.56	4	6.25
E. faecium	8	>128	100	>	n/a
P. aeruginosa	>32	8	n/a	>	100

All are MIC_{90} in µg/ml and are taken from literature references. n/a = not available. MRSA = methicillin-resistant S. aureus; MR-CNS = methicillin-resistant coagulase negative Staphylococcus; GISA = glycopeptide-resistant *S. aureus*; PRSP = penicillin-resistant *S. pneumoniae*.

E. faecium (27). The spectrum of activity against Gram-negative strains was most similar to that of cefepime and does not include *Pseudomonas* (28). Allometric modeling of the compound using five species allowed for the prediction of a 2-3h human half-life (29). One new cephalosporin, ME1209, **8**, demonstrated activity

against selected species of MRSA and ceftazidime-resistant *P. aeruginosa* (30, 31).



9

A new broad spectrum, bactericidal, parenteral 2-oxaisocephem OPC-20011, **9**, with improved binding to penicillin binding proteins was described and had promising activity *in vitro* against methicillin sensitive *S. aureus* (MSSA), MRSA, MRSE, ampicillin-resistant *S. pneumoniae*, and *E. faecalis* and had good activity *in vivo* (32).

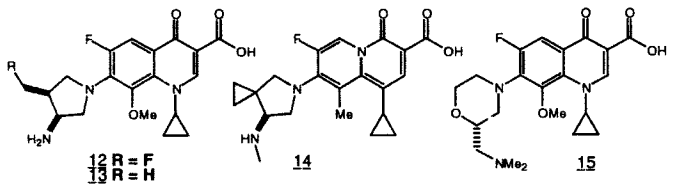
This past year the results of continued profiling of previously disclosed β-lactams were also reported. The results included recent clinical studies of the non-ester oral cephalosporin S-1090 against many infections including: RTI (33), UTI (34), skin and soft tissue (35), surgical and gynecological infections (36). *In vitro* examination of clinical isolates from those trials showed no resistance development during the trials and additional *in vitro* data against pathogens that cause obstetric and gynecological infections (37,38) were released. This cephalosporin exhibits enhanced stability to DHP-1 yet retains activity superior to imipenem against PRSP (39). Additional *in vitro* and *in vivo* properties of MC-02,479/RWJ-54428, **11**, were revealed, and the link between the possible association between binding to multiple PBPs and low frequency of resistance was investigated (40-43). New information on human multiple dose pharmacokinetics of S-4661 has been reported (44). In 1998, additional profiling of CS-834 continued to be reported. Included were: pharmacodynamic, pharmacokinetic, and murine efficacy studies against PSSP and PRSP (45-47). The anti-pneumococcal activity and activity against Neisseria spp. of penem MEN 10700 were also presented (48-51).

Glycopeptides - In recent studies of LY333328, healthy volunteers received single iv doses of 0.5 to 3 mg/kg with the compound exhibiting an extremely long $t_{1/2}$ of 132-356 h. The initial safety profile suggested that the compound was well tolerated at these single doses (52). The synthesis of and SAR around this class of antibiotics including LY333328 was published (53).

Quinolones - A comprehensive review of the chemistry, SAR and mechanism of action of quinolones was published (54). Since the last review, several late-stage clinical candidates continued to progress through clinical trials (55). Trovafloxacin and its iv companion alatrofloxacin gained US FDA approval and were launched in late January 1998 (56-59). Gatifloxacin, a broad spectrum 8-methoxy fluoroquinolone, completed Phase III clinical trials and an NDA was filed recently for both oral and iv forms (60-64). Clinafloxacin (iv only) and moxifloxacin (formerly known as Bay 12-8039, oral and iv) have also completed Phase III trials (65-72). SB-265805 (formerly LB-20304a), is in Phase III trails and reported to possess excellent activity against resistant *S. pneumoniae* strains (73-76). DC-756, **12**, was reported to possess potent activity against Gram-positive and Gram-negative pathogens comparable to trovafloxacin, with MIC_{90} against ofloxacin-resistant strains 16-fold better than trovafloxacin (Table 3). More importantly, the addition of a fluorine atom to the exocyclic methyl group alleviated the toxicity in the

micronuclei-formation test seen with the corresponding methyl analog **13**. DC-756 is well absorbed orally in rats and found to have good photostability (77-79). The half-life of **12** in the monkey is 2 h and a rather low urinary recovery (9-10% parent drug in rat and monkey) of the compound was observed. The discovery of A-170568, **14**, a 2-pyridone agent with a 3-methylamino-4-spirocyclopropyl pyrrolidine side chain was reported. Like other 2-pyridone analogs, **14** displayed potent *in vitro* and *in vivo* activity against a range of bacterial organisms. The oral efficacy of A-

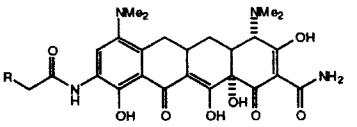
Table 3



	MIC$_{90}$ (trovafloxacin)	
	13	**14**
MSSA	0.03 (0.03)	0.015 (0.03)
QR-MRSA	1 (16)	0.5 (2)
PRSP	0.06 (0.5)	0.06 (0.25)
E. faecalis	4 (32)	0.25 (1)
E. faecium	4 (16)	n/a
E. faecium-van-R		1 (1)
QR-P aeruginosa	64 (>128)	16 (>128)

All are MIC$_{90}$ in μg/ml and are taken from literature references. n/a = not reported

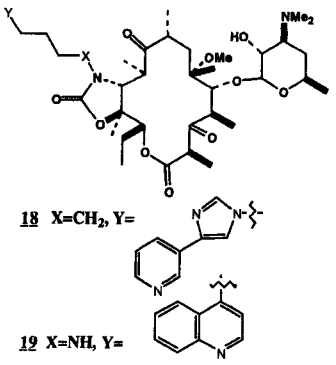
170568 in a mouse model was less than that of trovafloxacin versus *S. aureus, E. coli* and *P. aeruginosa*, with equivalent efficacy against *S. pneumoniae*, while its MIC$_{90}$s versus resistant organisms are improved over those of trovafloxacin. Compound **14** exhibited relatively low toxicity in an acute toxicity model in mice (LD$_{50}$ =232 mg/kg ip) and in the *in vitro* human topoisomerase II cleavage assays with an IC$_{50}$ versus human topoisomerase II of >100 μg/ml. It also demonstrated good oral bioavailability in rat (46%) and dog (96%). This compound is currently undergoing preclinical studies and may be the first in the 2-pyridone class to enter human trials (80). Another novel quinolone agent, Y-34867 (**15**), was reported to have excellent *in vitro* and *in vivo* activity against *Helicobacter pylori*. This compound demonstrated higher potency than amoxicillin and clarithromycin against *H. pylori* in mice and Mongolian gerbils. In addition, in combination with famotidine, it showed a profound synergistic effect against *H. pylori* in Mongolian gerbils (81).

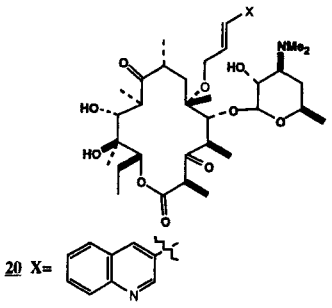


16 R = tBuNH
17 R = pyrrolidinoglycylamido

Glycylcyclines - This past year there were two reviews of the glycylcyclines (9, 82). Two new glycylcyclines, 9-t-butylglycylamido minocycline (TBG-MINO, or GAR-936, **16**) and 9-pyrrolidinoglycylamido minocycline (PAM-MINO, **17**) were described. GAR-936 demonstrated broad spectrum antibacterial activity *in vitro* and was effective against a wide range of pathogens, including MRSA, PRSP and VRE, in murine models (83). It is also efficacious in the treatment of experimental endocarditis in rat (84). GAR-936 was found to be a poor substrate for the efflux pump and it also appears to be quite difficult to develop resistance to this compound in the laboratory (85, 86).

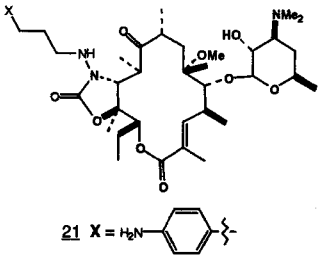
Macrolides -The major thrust in the macrolide area continues to be the development of ketolides (87). HMR-3647 (**18**) is currently in Phase III clinical trials and some of the Phase I results were published (88). In addition, numerous reports on *in vitro* and *in vivo* activities of this compound were published (89-92). The chemistry and the conformational analysis of its predecessor, HMR-3004 (**19**) were also discussed (93, 94).

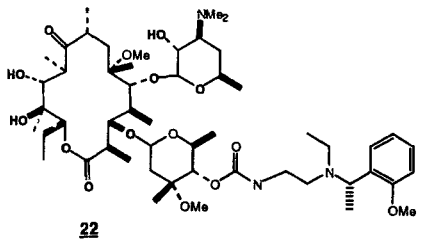




A new series of ketolides in which an arylalkyl group is attached to the C-6 hydroxy group of erythromycin A was disclosed (95, 96). For example, A-184656 (**20**) was active against both MLS_b resistant *S. aureus* and resistant *S. pneumoniae via* an efflux mechanism.

Other non-ketolide, des-cladinose analogs were investigated (97-99). Compounds of this class, represented by structure **21**, were potent *in vitro* but showed only moderate *in vivo* activity. Ketolide congeners of azalides were very recently disclosed by two groups (100, 101).

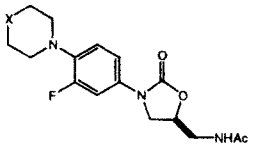




Potent non-ketolide analogs were developed by modifying the C"-4 hydroxy group of macrolides (102-106). This series represented by CP-544,372

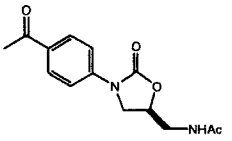
(**22**) had activity against macrolide resistant *S. pneumonia* (MIC = 0.04 - 0.2 μg/ml). Compound **22** exhibited *in vivo* activity similar to HMR-3647. In recent years, effort has been made to generate novel macrolides by recombinant polyketide synthesis (107,108). Precursor feeding to such an organism has provided novel C-13 erythromycin analogs (109).

Oxazolidinones - Linezolid (PNU-100766, **23**) is currently in Phase III clinical trials and some pharmacokinetic data has been published (110-114). Concerning the mechanism of action, a recent report indicates that linezolid inhibits formation of ternary complex of N-formyl-methionyl-*t*RNA-ribosome-*m*RNA (115). DuP721 (**24**) is reported to inhibit the puromycin-mediated release of S-formyl-methionine from 70S initiation complex (116).



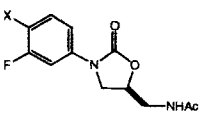
23 X=O

27 X= N-(C=O)CH_2OH



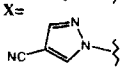
24

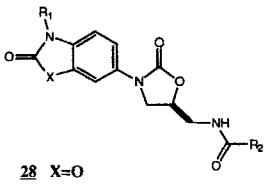
A series of novel oxazolidinones in which the distal nitrogen is replaced by a 5-membered heteroaromatic ring was reported as an extension of the initial work. PNU-107922 (**25**), for example, had good activity against *S. aureus* and *S. pneumoniae* (117). Cyanopyrazole analog PNU-172576 (**26**) was found to be approximately three times more potent *in vivo* than linezolid (118). These compounds are also active against *H. influenzae* and *M. catarrhalis*, and as such, represent the first oxazolidinones with potentially useful activity against Gram-negative pathogens (119). Attachment of 6-membered heterocycles also provided some analogs whose *in vitro* and *in vivo* activity was comparable to that of eperezolid (**27**) (120). A new series of oxazolidinones, which possess fused ring systems, was reported. These compounds (**28** and **29**) are reported to exhibit greater potency (MIC range 0.25 - 2 μg/ml) than linezolid (121-123).



25 X=

26 X=





28 X=O

29 X=S

Miscellaneous - Synercid (RP59500) received US approval for severe Gram-positive infections including vancomycin-resistant *E. faecium* (124). Ziracin (Sch-27899), an injectable everninomycin oligosaccharide antibiotic with low nephrotoxic potential, is in clinical Phase III trials against Gram-positive infections. It is active against MRSA, VRE and PRSP strains (125-132). Daptomycin, a synthetic derivative of the acidic lipopeptide A21978C, belongs to the cyclic lipopeptide class of antibiotics and inhibits cell wall synthesis. It is currently undergoing through

Phase III clinical trials targeting serious hospital infections, including those caused by MRSA and VRE in iv formulation (133-136).

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SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

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Chapter 18. Inhibitors of Integrin Alpha 4 Beta 1 (VLA-4)

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Introduction - The α4 integrins, α4β1 (VLA-4) and α4β7, are heterodimeric cell-surface proteins expressed on leukocytes, involved in both cell-cell and cell-matrix interactions. These integrins are key regulators of physiologic and pathologic responses in inflammation and autoimmune disease. Their central role in animal models of inflammatory disease has been extensively documented using α4-specific monoclonal antibodies. Integrins are proven, valid targets for small molecule drugs as is well documented by the development of both parenteral and orally active antagonists of platelet integrin αIIbβ3 and, more recently, integrin αVβ3. Therefore, there is a significant effort in many laboratories to develop small molecule inhibitors of α4 integrins for immunoinflammatory and autoimmune diseases. Early animal model work that validated α4 in disease has been thoroughly reviewed (1). More recently, the biology of α4 integrins in asthma has been reviewed, as has early work on the chemistry of α4 integrin antagonists (2). Herein we summarize recent advances in the α4β1 arena with particular emphasis on the chemistry of small molecule antagonists, and on inflammation as a clinical target.

RECENT BIOLOGY

The α4β1 integrin is expressed at high levels on mononuclear leukocytes, mast cells, macrophages, basophils, and eosinophils. Although originally thought not to be expressed on polymorphonuclear leukocytes (PMNs), recent studies suggest functional α4 can also be expressed on PMN in vitro when treated with cytochalasin and inflammatory mediators such as C5a or fMLP (3). Physiologic stimuli equivalent to cytochalasin have not yet been identified; however, trans-endothelial migration of human PMN across endothelial cell monolayers in response to C5a renders the PMN competent for fMLP-induced expression of α4β1 from granule stores (4). Furthermore, emigrated rat PMN isolated from the peritoneal cavity following thioglycolate induction express α4 and can be induced to express 5-fold higher levels with fMLP (5). These data suggest, in contrast to earlier conclusions, that α4 may play an important role in PMN function particularly following emigration into tissues. The pathophysiologic significance of these results in human asthma requires further study, since PMN are recruited into the lung in animal models of allergic lung disease and in human asthma (6,7).

VCAM-1 and the alternatively-spliced type III connecting segment-1 (CS-1) of fibronectin (Fn) have been the only native ligands for α4β1 characterized to date. Recent work suggests that α4β1 also binds another matrix ligand, osteopontin, a highly post-translationally modified phosphoprotein with multiple functions (8). Osteopontin's role as an α4β1 ligand may be very important as it is strongly upregulated in many inflammatory settings, including inflamed lung (9).

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CHEMISTRY

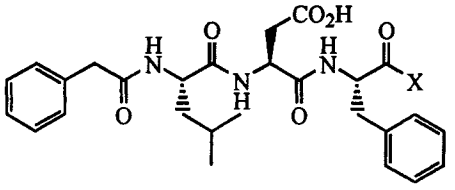
Some medicinal chemistry on α4β1 has recently begun to appear in the refereed literature. However, owing to the early stage of drug development in the field, recourse to the patent literature is required for a sense of recent progress. A subset of recent patent filings allow fairly reliable determination of preferred compounds from in vitro properties, and some supportive biology is also available.

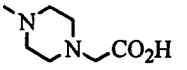
It was discovered during early cell adhesion studies with α4β1 that the integrin binds Fn CS-1 and VCAM-1, through short contiguous peptide sequences within each ligand. The minimal recognition sequence in CS-1 was determined to be LDV (10,11), while the recognition domain within VCAM-1 was found to be IDS (12). Synthetic versions of these peptides were effective inhibitors of α4β1-mediated cell adhesion when added in solution to the adhesion milieu. The minimal peptides were very weak inhibitors with IC_{50}s greater than 1 mM; however improvement in potency was achieved by extending the peptides from the C- and the N-termini, in the case of Fn (EILDVPST), or by extending and cyclizing through disulfide linkage in the peptide derived from VCAM-1 (cyclic-CQIDSPC). The initial success with simple peptide inhibitors encouraged a number of investigators to undertake the development of peptide analogs and peptide mimetics, and to date compounds with $>10^6$-fold improvement in affinity for α4β1 have been discovered that exhibit efficacy in animal disease models. These findings are important because, in most instances, the compounds achieve levels of efficacy similar to the maximal efficacy seen with antibodies which are effectively irreversible receptor antagonists. These results suggest that antagonism of highly avid protein-protein interactions, inherent in cell adhesion processes, can be achieved with small molecules. While many of the early compounds are not very potent in the animal models, recent results portend likely future advances.

Linear peptides and peptide analogs - The earliest work in the search for α4β1 inhibitors focussed on simple linear analogs of the prototype LDV sequence. A family of N- and C-terminally capped LDV analogs that improved upon the prototype peptide, GPEILDVPST, by nearly 3000-fold has been patented (13-15). Compounds **1a-c** capped N-terminally with phenylacetyl and at the C-terminus by D-proline, morpholine or 1-piperazinyl-4-acetic acid amides represent optimal analogs from the series.

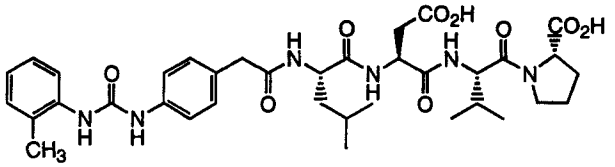
These compounds have played an important role in the α4β1 story as they have been used to extensively confirm animal model data originally obtained with mAbs. The D-proline amide **1a** was employed in a mouse model of oxazolone-induced contact hypersensitivity at a dose of 6 mg/kg s.c. via pumps where the overall efficacy was comparable to the anti-α4 mAb PS/2 (13,16). The same compound was also effective in a hyperlipidemic rabbit heterotopic heart allograft model (17). In this model, an HLA-mismatched donor heart was transplanted into the neck of a recipient animal without immunosuppression, and after 7-8 days the heart experienced functional decline incident to graft rejection. Graft failure was accompanied by massive inflammatory cell influx associated with ICAM-1, VCAM-1 and Fn upregulation and by intimal thickening and luminal narrowing of the coronary arteries, especially in small and medium sized vessels. Active compound **1a**, or an inactive, scrambled control, was administered daily at 1 mg/kg s.c. beginning on the day of transplant, and animals were sacrificed after 7-8 days for histological examination. Treatment with active drug, but not inactive control, essentially normalized the extent and the severity of coronary vessel arteriopathy. Moreover, inflammatory cell influx and increases in the synthesis of cell adhesion molecules were significantly inhibited. Additionally, **1a**

inhibited late-phase bronchoconstriction and airways hyperreactivity in naturally allergic sheep that were challenged with nebulized *Ascaris* antigen (18). Nebulized drug, or inactive control compound, was administered twice daily for three days at 1 mg/kg as well as 30 minutes before and 4 hours after antigen challenge on day four. Non-specific airways hyperresponsiveness to carbachol challenge 24 hours after antigen challenge was also inhibited. The study was conducted as a double cross-over design with a rest-period between each study to allow hyperresponsiveness to normalize, thus each animal served as it's own control. Further underscoring the generality of the role of α4β1 in allergic airways models, the morpholine amide **1b** also proved effective in a rabbit airways challenge model using house dust mite antigen as immunogen. In this case, a single 100 mg/kg nebulized dose given 15-30 minutes before antigen challenge inhibited early and late bronchoconstrictor responses and normalized dynamic compliance during the 6 hour test period. Again, the study was conducted as a double cross-over design using an inactive control compound (14).



		IC_{50} (μM)
1a	X =	2
1b	X =	0.2
1c	X = 	0.08

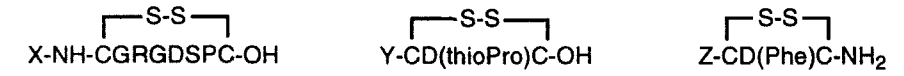
A series of N-terminally capped LDV peptide analogs was concurrently developed. Using a combination of cell adhesion assays and a binding assay based on a bivalent immunoglobulin heavy chain fusion protein with the two terminal Ig domains of VCAM-1 (VCAM-Ig), a series of potent, tight binding inhibitors was discovered (19,20). A prototype peptide, YLDV (IC_{50} = 50 μM, cell adhesion) served as the starting point with optimization focussed on the tyrosine residue. BIO-1211 **2** with the novel 2-methylphenyl urea phenylacetyl functionality proved to be optimal. Compound **2** inhibited binding of VCAM-Ig to α4β1 as well as cell adhesion to CS-1 with IC_{50} values of 1-5 nM. Radiolabelled **2** was also utilized in direct binding studies with Jurkat cells and with human blood lymphocytes where the compound bound with >100-fold selectively to the activated form of α4β1 with K_D = 70 pM, and it exhibited slow dissociation kinetics with a half-time of about 90 minutes. The compound was also highly selective relative to alternative integrins; the homologous α4β7 was the only other integrin bound, however with 1000-fold lower affinity. Additionally, **2** appeared to be a true LDV-mimic in that ligand-induced epitopes appeared on α4β1 with a concentration-dependence identical to binding. Compound **2** was studied in the same allergic sheep model discussed above; however, in this case, the compound was >10-fold more potent than **1a** as a single nebulized dose of 0.1 mg/kg given 2 hours before antigen challenge. It completely blocked late-phase airways responses and normalized hyperresponsiveness at 24 hrs.



2

Cyclic Peptides—Operating on the well established precedent that conformational constraint of flexible peptide ligands can impart substantial enhancement to binding affinity, a number of investigators have undertaken the preparation of cyclic peptide inhibitors of α4β1 to improve upon results obtained with early peptide inhibitors.

Starting with cyclic RGD-containing peptides that weakly inhibited cell adhesion to fibronectin, two classes of cyclic peptides that are dual inhibitors of α4β1 and α5β1 were identified (21). The first series comprises octapeptide disulfides cyclized through terminal cysteine residues (22). Compounds **3a,b** exemplify this series; the unsubstituted compound **3a** inhibited α4β1-mediated adhesion to CS-1 with an IC_{50} of 20 μM, while the N-terminal analog **3b** was 10-fold more potent. Interestingly, both compounds were equipotent (IC_{50} ~ 80 μM) in blocking α5β1 adhesion to the 120 Kd fragment of Fn, a specific α5β1 ligand. The second series comprised highly constrained cyclic tetrapeptides, cyclized, again, through a disulfide linkage (23). The most potent analog **4a**, as in the first series, bound to both α4β1 and α5β1 which was apparent from specific adhesion assays and from affinity absorption experiments. Compound **4a** inhibited adhesion to CS-1 with IC_{50} = 3 μM. Additionally, the patent literature suggests that N-terminal substitution with hydrophobic groups enhanced activity as in the first series; compound **4b**, for example, inhibited cell adhesion to CS-1 with IC_{50} of 0.1 μM (24).



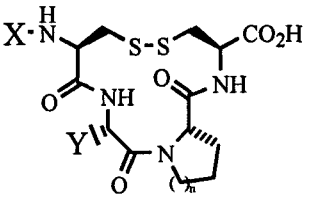
3a X = H
3b X = 1-adamantylacetyl

4a Y = Arg
4b Y = 1-adamantylacetyl

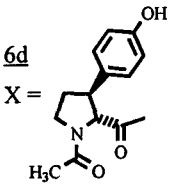
5a Z = H
5b Z = phenylacetyl

A homologous cyclic tetrapeptide in the context of an optimal linear analog series was identifed by systematically substituting two different, non-contiguous amino acids with cysteine and then cyclizing through a disulfide linkage. As in the linear series, a hydrophobic phenylacetyl group at the N-terminus was preferred and the proline, which was central to this series, could be substituted with phenylalanine, as exemplified by **5b** which exhibited an IC_{50} of about 0.3 μM (25).

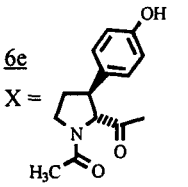
The cyclic tetrapeptide series was extended by systematically varying amino acids in each position (26). It was found that tyrosine or N-acetyl tyrosine served well as the N-terminal cap **6a**. On the other hand, the aspartate side chain, long thought to be essential in LDV mimics, was dispensable in this series, as long as the C-terminal cysteine was the carboxylic acid. The serine analog **6b** was preferred in the N-acetyl tyrosine series. Moreover, substitution of proline by pipecolic acid, expanding the proline ring by one carbon, imparted a 10-fold improvement in affinity as exemplified by **6c**. Further optimization of the N-terminal residue with a conformationally constrained tyrosine analog provided **6d** with an IC_{50} improved to 8 nM. Importantly, when each of these individual improvements were combined in a single analog, additive effects were observed; the optimal analog **6e** blocked cell adhesion with an IC_{50} of 0.5 nM. In an in vivo mouse model of cellular recruitment, compound **6b** blocked approximately 50% of ^{51}Cr-labelled mesenteric lymph node cells that home to Peyer's patches when it was co-administered i.v. at 25 mg/kg. The anti-α4 mAb PS/2 blocked >95% of homing where sufficient PS/2 was given to saturate both α4 integrins. It is now well established that lymphocyte homing to gut lymphoid tissue occurs through α4β7-MAdCAM interactions and only minimally through α4β1 (27); thus, this result with **6b** may reflect activity at α4β7 rather than α4β1. The integrin selectivity of **6b** was not reported in the paper.



	X	Y	n	IC_{50} (nM)
6a	Tyr	CH_2CO_2H	1	400
6b	Ac-Tyr	CH_2OH	1	120
6c	Ac-Tyr	CH_3	2	20



Y = CH_2OH
n = 1
IC_{50} = 8 nM



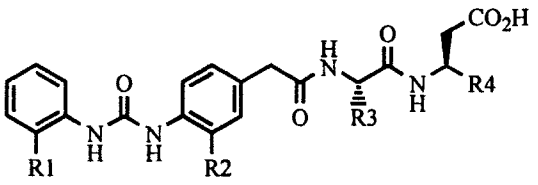
Y = CH_2OH
n = 2
IC_{50} = 0.5 nM

A similar systematic positional exchange of amino acids within a cyclic LDV peptide sequence delivered a potent hexapeptide, cyclic-CWLDVC **7** (28). This compound inhibited cell adhesion to CS-1, VCAM-Ig, and to IL-1 stimulated human umbilical vein endothelial cells (HUVEC) with an IC_{50} of 0.1-10 μM, comparable with other cyclic peptides described above. It also inhibited α4β7-specific cell adhesion, however the IC_{50} was 10-fold greater suggesting modest selectivity for α4β1. On the other hand, and in contrast to some cyclic peptides, this compound did not bind to α5β1.

Peptide Mimetics - Early peptide studies on α4β1 focussed on identifying leads suitable for further development as medicinal agents. Developing compounds beyond peptides is particularly important for α4β1 inhibitors because the role of α4β1 in animal models clearly points toward chronic inflammatory disease targets requiring oral dosage for effective clinical compliance. Thus, much of the cyclic peptide work described above, in addition to providing reagents for biological studies, has sought to identify high affinity conformationally rigid constructs from which pharmacophore models could be developed as templates for drug design and for screening compound libraries. While direct results from this approach have not yet been described for α4β1, success by this approach in the αIIbβ3 field suggests it should be possible.

On the other hand, the classic peptide mimetic approach has begun to bear fruit. With the discovery of the optimal tyrosine replacement described above in **2**, a variety of peptide mimetic strategies to replace the tetrapeptide was explored. One series, based on β-amino acid dipeptide mimics, recapitulated the potency in binding and cell adhesion assays of the original peptide analog **2**. Representative analogs in the series **8a-h** inhibited VCAM-Ig binding to α4β1 and cell adhesion to CS-1 with IC_{50}s in the low nanomolar range (29). In general, the SAR at each position in the molecule could be optimized independently and improvements were additive when combined in a single molecule. Moreover, potent molecules from this series were active in a

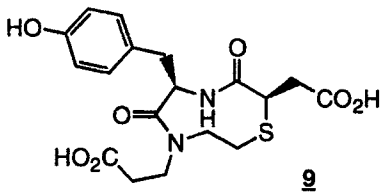
mouse contact hypersensitivity model and in the sheep model of allergic airways disease.



	R1	R2	R3	R4	IC_{50} (nM) binding	adhesion
8a	H	H	i-Bu	1,3-benzodioxol-5-yl	12	50
8b	H	OCH_3	i-Bu	1,3-benzodioxol-5-yl	-	3
8c	CH_3	H	i-Bu	1,3-benzodioxol-5-yl	1	2.5
8d	CH_3	H	i-Bu	H	27	-
8e	CH_3	H	i-Bu	CH_3	2	7
8f	CH_3	H	$CH_3S(CH_2)_2$	1,3-benzodioxol-5-yl	-	3
8g	CH_3	H	i-Bu	$-CH_2CHCH_2$	0.3	-
8h	CH_3	H	H	$-CH_2CHCH_2$	35	-

Peptide Mimetics from Screening Leads – A number of patent applications have recently appeared (31-44). One surmises that the general dipeptide and tripeptide mimetic motif resides in a large number of screening libraries and that they been productively employed on α4β1. Alternatively, the relative ease with which parallel array and combinatorial libraries can now be synthesized may have provided rapid entrée to these series. Future publications from these investigators should clarify the actual strategies employed.

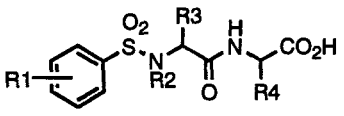
The first published report on combinatorial solid-phase synthesis directed toward α4β1 recently appeared (30). Constrained, cyclic sulfide β-turn scaffolds were the focus of the study with pendant functionality for this library based on α4β1 preferences inferred from the cyclic hexapeptide **7**. Compound **9**, a preferred compound from this initial effort blocked cell adhesion to CS-1 with IC_{50} = 5 μM.



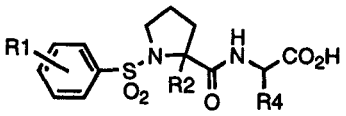
9

Arylsulfonyl-Pro-Phe mimetics—A class of arylsulfonylated dipeptide mimetics based on the Pro-Phe sequence have been identified (31). Though no biological data was mentioned, apparent preferred compounds in the first series involve phenyl and substituted phenyl sulfonamides of hydrophobic dipeptides as exemplified by **10**. In general, hydrophobic residues in both amino acid positions appear to be preferred. In a second related series exemplified by **11**, the first amino acid was replaced by proline or the homologous 4- or 6-atom homologues (32,33). Moreover, fused ring analogs

comprising isoindole or tetrahydroisoquinoline substitutions, and hydroxy or amino substituted proline were also tolerated. As in the first series, a wide range of amino acid side-chains were incorporated at the second amino acid position with hydrophobic groups apparently preferred.



10

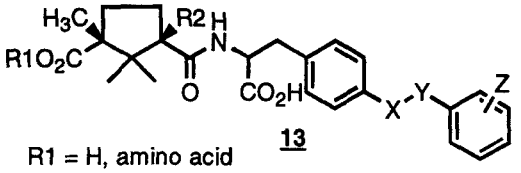


11

R1	R2	R3	R4
H	H	alkyl	C_4H_9
halogen	CH_3	aryl	2-naphthylmethyl
CF_3		arylmethyl	4-fluorophenylmethyl
OCH_3			4-biphenylmethyl

Pro-Tyr benzylether mimetics - A protected dipeptide series focussed on the 2,6-dichlorobenzyl ether of N-acetyl-(D)-thioprolyl-tyrosine **12** (34). No activity data was reported.

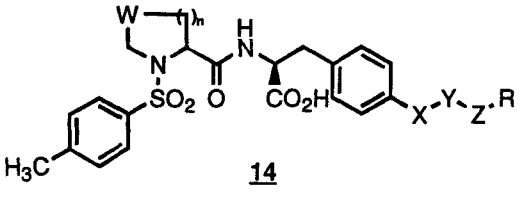
Cyclopentyldicarboxylicamide-Phe and -Tyr mimetics - A dipeptide mimetic series **13**, incorporating 2,2,3-trimethylcyclopentane dicarboxylate as a proline mimic was discovered (35). The best compounds in this series incorporated halo-substituted benzyl groups on tyrosine or halobenzoyl functionalized p-aminophenyl alanine with low nanomolar IC_{50} values.



13

R1 = H, amino acid
R2 = H, CH_3
X = O, N Y = H_2, CO Z = halogen

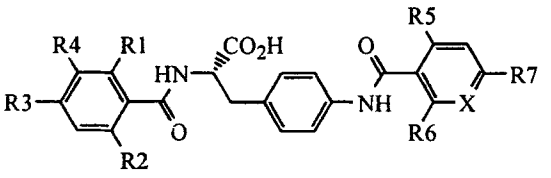
Arylsulfonyl-dipeptide mimetics - A series of patent applications detail arylsulfonylated dipeptide mimetics **14** closely related to the arylsulfonyl-Pro-Phe series noted above (36-42). These applications focussed on proline and thioproline in the first amino acid position and on 4-substituted phenyl alanine analogs in the second position. They incorporated an extensive array of functionality at the 4-position with particular emphasis on aryl- and alkyl-substituted amides, urethanes and ureas. Biological data was not reported, however hydrophobic functionality appeared to be preferred.



14

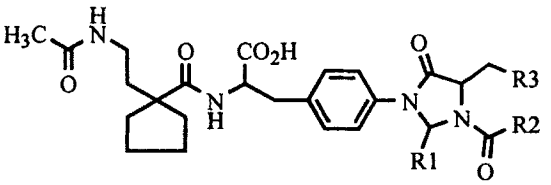
W = CH_2, S
X = N, O
Y = CO, CS, CH_2
Z = N, CH_2, 1-morpholine,
1-piperazine (R = 4-H, 4-Boc)
R = aryl

Aryl diamide analogs of p-amino-Phe - An interesting analog series based on p-aminophenyl alanine was disclosed (43). The best compounds of this series incorporated different substituted N-benzoyl functionalities at both the α- and the p-amino groups. Optimal compounds incorporated halogen and/or alkyl substitution on both benzoyl groups as exemplified in **15a-g**.



	R1	R2	R3	R4	R5	R6	R7	X	IC_{50} (nM)
15a	Cl	CH_3	H	H	Cl	Cl	H	CH	15
15b	H	Br	H	OCH_3	Cl	Cl	H	CH	20
15c	CH_3	i-Pr	H	H	Cl	Cl	H	CH	23
15d	Br	CH_3	H	H	Cl	Cl	H	CH	9
15e	Cl	H	CH_3SO_2	H	Cl	Cl	H	CH	9
15f	Cl	CH_3	H	H	H	CH_3	CH_3	N	20
15g	H	Br	H	OCH_3	H	CH_3	CH_3	N	22

Finally, a second series on the 4-aminophenylalanine theme **16**, that incorporated a novel cyclopentane proline substitution also afforded compounds with low nanomolar IC_{50} values (44). It is interesting that the 4-acylamino when incorporated as the constrained cyclic heterocycle supports impressive potency. Once again, halo-substituted aryl functionality at the "right-hand side" of the molecule continues the theme of hydrophobicity being preferred in this family of α4β1 inhibitors.



	R1	R2	R3	IC_{50} (nM)
16a	Ph	CH_3	4-OMe-Ph	2
16b	Ph	CH_3	3-pyridyl	2
16c	Ph	CH_3	Ph	3
16d	p-Cl-Ph	C_3H_7	Ph	5
16e	p-Cl-Ph	C_3H_7	4-OMe-Ph	<10

Conclusions - Since the discovery of it's key role in mediating inflammatory pathophysiology, α4β1 has received considerable attention as a drug design target. Important advances have been made in identifying potent and selective candidates for further development strongly suggesting that α4β1 should be a tractable small molecule target. Most importantly, biological evaluation of advanced compounds suggests that efficacy comparable to that achieved with mAbs is feasible which augers well for eventual clinical and commercial success in this emerging drug development arena.

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Chapter 19. Glucagon and Glucagon-Like Peptide-1

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Introduction – Glucagon and glucagon-like peptide-1 are hormones generated from *pro*-glucagon, the product of the glucagon gene (1). The expression of *pro*-glucagon is primarily in the pancreas and the gastrointestinal tract. Post-translational processing of *pro*-glucagon in pancreatic α-cells specifically produces glucagon, whereas GLP-1 is primarily produced by post-translational modification in L-cells of the lower intestine (2) (Figure 1). Intestinal processing of *pro*-glucagon produces a variety of proteins all of which are secreted into the peripheral circulation in response to intake of carbohydrates and other nutrients. One protein, GLP-2, has recently been shown to stimulate intestinal growth (3); the biological activities of the other proteins are unclear (4).

Glucagon and GLP-1 both provide important controls on intermediary metabolism, although through very different physiologic processes. Both hormones are targets for the development of new therapies for the treatment of Type II diabetes, based on their respective roles in the regulation of glucose metabolism and insulin secretion (5).

Glucagon Physiology - Glucagon is normally secreted by α-cells in pancreatic islets in response to falling blood glucose levels (6). The hormone binds to a specific receptor in liver that triggers glycogenolysis and an increase in gluconeogenesis through cAMP-mediated events (7,8). These responses generate glucose (e.g. hepatic glucose production) to help maintain euglycemia by preventing blood glucose levels from falling significantly below 4 mM.

In Type II diabetic subjects, glucagon secretion is abnormal, leading to inappropriately high plasma glucagon concentrations under conditions of elevated blood glucose (9). Another consideration is the resistance to insulin action that is present in Type II diabetes (10). Because insulin opposes glucagon action, the insulin resistance magnifies the glucagon effects on hepatic glucose production. Studies of Type II diabetic subjects that have directly measured hepatic glucose output have shown a causal role for glucagon in excessive glucose production (11). Animal studies that used antibodies against glucagon or peptides that block glucagon binding have also supported a role for glucagon in maintaining elevated blood glucose (12,13). These findings argue that a therapy based on blocking glucagon action will provide an important advance in the management of Type II diabetes.

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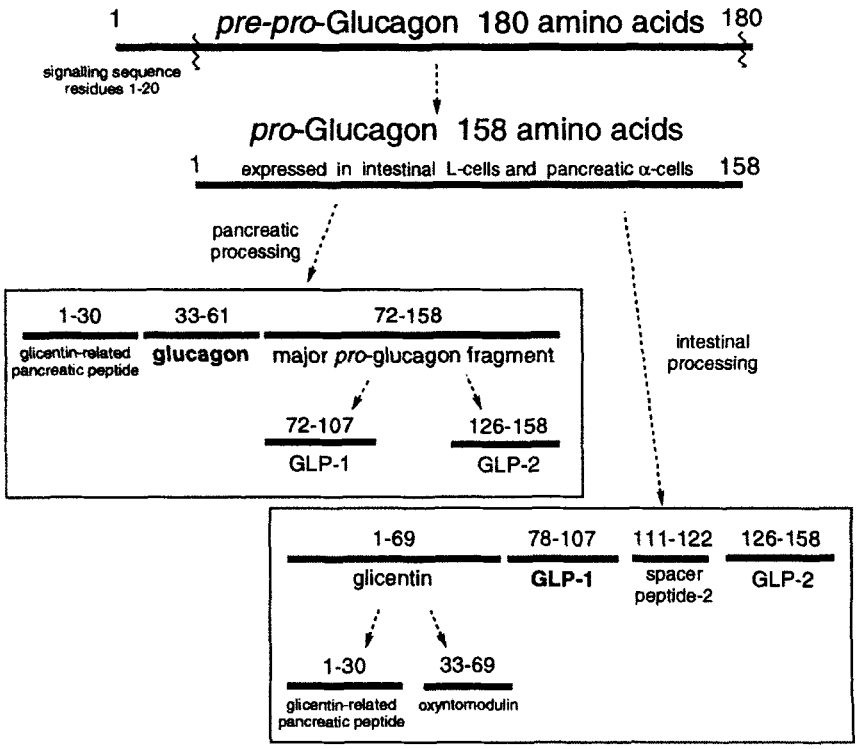


Figure 1. pro-Glucagon Processing In Vivo

GLP-1 Physiology – By comparison, GLP-1 is being evaluated as a treatment for the abnormality in insulin secretion found in Type II diabetes (10,14). Two forms of GLP-1, GLP-1 (7-37) and GLP-1 (7-36 amide), are produced by L-cells in the jejunum and colon in response to oral glucose or mixed meals (15). GLP-1 increases glucose-mediated insulin secretion by activating specific GLP-1 receptors on insulin secreting β-cells in the pancreatic islets (16). The increase in insulin secretion is glucose-dependent and stems from increases in cAMP and Ca^{2+} levels. This benefits Type II diabetic subjects by improving the β-cell's response to glucose and thereby increasing insulin secretion (17,18). Based on these findings, the relevant antidiabetic therapy is a drug that enhances the GLP-1 effects or elicits a GLP-1-like action in the β-cell.

GLUCAGON RECEPTOR ANTAGONISTS

Glucagon Receptor – Most attempts to find agents that block glucagon action have usually targeted the glucagon receptor. This G-protein-coupled receptor has been cloned and sequenced (8) and shown to be a single gene that does not have deferentially spliced variants (19). The glucagon receptor belongs to a subfamily that includes receptors for secretin, VIP, GLP-1, PACAP, GRF, calcitonin and parathyroid hormone (20). The glucagon binding site was mapped by creating chimeric receptors with elements of the glucagon receptor and the closely related GLP-1 receptor (21). The mapping indicated a requirement for the proximal half of the amino-terminal

extension and the third, fourth and sixth transmembrane domains. Point mutations in other studies have shown the importance of the N-terminal domain for glucagon binding activity (22). Residues 252 to 259 in the second intracellular domain of the receptor produce the divalent cation effects on glucagon binding (23). Although information is growing on glucagon-glucagon receptor association, the structural information needed to predict the design of an antagonist is not at hand.

Peptide Antagonists - The first glucagon antagonists were peptide mutations of glucagon. One of the earliest antagonists is *des*-His^{1} glucagon (24). However this peptide is not a full antagonist; it retains a weak ability to activate the receptor. By 1992 over 100 glucagon analogs had been prepared and random mutagensis has provided another 3500 analogs for evaluation (25). Findings from analoging studies indicate that the N-terminus of glucagon is involved in receptor activation and partially aids in receptor binding. In contrast, the carboxyl-terminal region is involved primarily with receptor binding and a hinge region (amino acids 9-14) that allows the two domains to communicate (19,26,27).

The indistinct separation of the binding and activation domains on glucagon presents a challenge for designing a highly active, full antagonist. Although a change in structure may render the peptide incapable of activating the receptor, the modification may also lower the affinity of the peptide for the receptor, resulting in a weak antagonist. N-Trinitrophenyl glucagon was among the earlier glucagon antagonists to be synthesized (28), which argued for the importance of N-terminal modifications to produce glucagon antagonism (29). Another improvement was the introduction of a C-terminal amide (30). Evaluation of a variety of analogs in the *des*-His^{1}-glucagon amide series identified more active antagonists. For example, *des*His^{1}-Glu^{9}-glucagon amide has about 40% of the binding affinity of native glucagon, but no signaling activity (30). This analog was able to suppress glycogenolysis in rabbits and reduce the hyperglycemia in diabetic rats when a 100-fold excess of the peptide over glucagon was administered (31).

N-trinitrophenylhistidine^{1}-homoarginine^{12}-glucagon is also a full antagonist and when infused, lowers the blood glucose of diabetic rats by 50% (13). Other interesting full antagonists are cyclic *des*His^{1} glucagon amides that are cyclized through Glu^{9} to Lys^{12} or Lys^{12} to Asp^{15} (32). The K_d for binding to the glucagon receptor was 1.5 nM for the Glu-Lys analog and 4 nM for the Lys-Asp derivative. Neither cyclized peptide produced any stimulation of cAMP production.

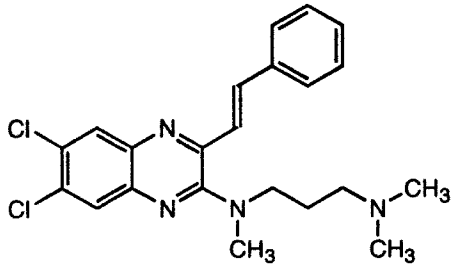
Random mutagenesis has been used to prepare large numbers of glucagon analogs of which over 20 are antagonists (25). As previously found, a change in one of the first four N-terminal amino acids is required for antagonism. Some additional increase in antagonism is provided by changes in amino acids 5, 7, 9, 11, 13, 21 and 29. The conservative replacement of a serine residue for threonine at position 29 provides a surprising increase in antagonism, underscoring the difficulty of predicting the effect of a particular amino acid substitution (25). However, none of the new analogs are any more potent antagonists than *des*His^{1}-Glu^{9}-glucagon amide.

One peptide antagonist of glucagon has been reported to be in preclinical development. This analog, *des*His^{1}-Nle^{9}-Ala^{11}-Ala^{16}-glucagon amide, lowers fasting blood glucose for a period of 5 hours in streptozotocin-induced diabetic rats by a maximum of 60% (33). The EC_{50} for this effect is 33 μg/kg when administered intravenously.

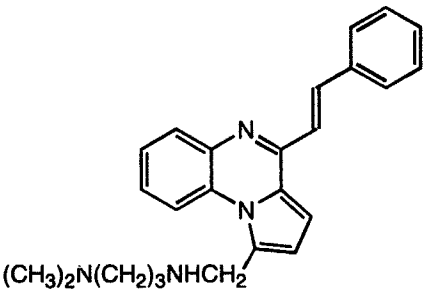
NON-PEPTIDYL GLUCAGON ANTAGONISTS

Non-peptide Antagonists – Successful cloning and expression of the glucagon receptor has enabled research groups to search for novel ligands as potential therapeutics. A growing number of structurally diverse non-peptide glucagon receptor antagonists have been reported in the scientific and patent literature.

In 1992, the first report of a non-peptide ligand for the glucagon receptor appeared (34). Identified by screening against the rat glucagon receptor, a weakly active quinoxaline compound 1 (CP-99,711) was found that binds to rat liver membranes with an IC_{50} of 4 μM. In a functional assay, compound 1 is a full antagonist and reduces glucagon-stimulated cAMP production with an IC_{50} of 7 μM. However, as the authors point out, quinoxaline 1 is not selective for the glucagon receptor and was found to bind to several other G-protein-coupled receptors. Recent studies with 1 indicate that it is a non-competitive inhibitor of glucagon binding (35).



1 CP-99,711



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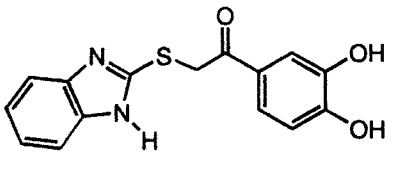
A modification of the core quinoxaline template of 1 by constraining the dimethylaminopropyl sidechain led to the identification of the structurally related pyrrolo-quinoxaline series. This series has also been reported to inhibit glucagon binding to its receptor (36). While retaining the styryl and dimethylaminopropyl sidechains of the quinoxaline compound 1, the pyrrolo[1,2-a]quinoxaline series shows antagonism of the glucagon receptor in the low micromolar range. The most potent analog 2 has an IC_{50} of 5 μM in a glucagon binding assay.

A series of phenolic compounds has been described as glucagon antagonists (35). These compounds are competitive antagonists with binding affinities to the human receptor in the micromolar range. Functional activity of the compounds tracks closely with the binding affinity. A number of analogs are reported of which the 4-hydroxy-3-chlorobenzoyl derivative 3 is the most potent with an IC_{50} of 12 μM in a binding assay. Related analogs in which the benzimidazole is replaced by benzthiazole or benzoxazole show greatly reduced affinity for the receptor (37).



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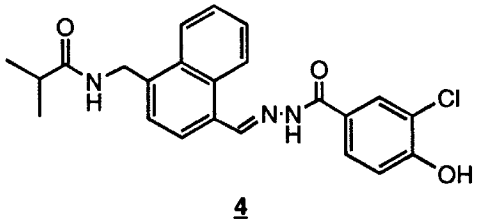
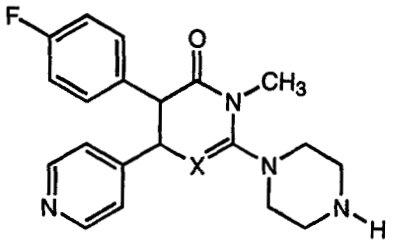


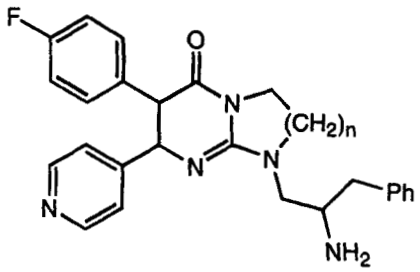
4

A related series of acyl hydrazide-based antagonists, also containing the 4-hydroxy-3-chlorobenzoyl group, is reported in the patent literature (38). A vast number of examples are disclosed in this application which focuses on disubstituted aryl derivatives, exemplified by the disubstituted naphthalene derivative **4**. No binding data or functional activity of the compounds are disclosed.

Pyrimidone compounds **5** and **6**, and related analogs (**7**) have been reported in the patent literature as glucagon antagonists (39). The compounds are competitive antagonists of the human glucagon receptor, but the data are insufficient to formulate structure-activity relationships. The compounds are not selective for the glucagon receptor and also have effects on TNF-α, interleukins and prostaglandin production.

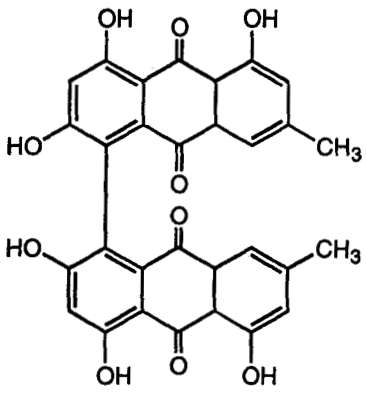


5 X = N
6 X = CH



7 n = 1-3

A glycoside-containing natural product extract with glucagon antagonist activity has been disclosed in the patent literature (40). The *bis*-anthraquinone skyrin **8**, a pigment isolated from lichens and fungi, is also claimed for anti-glucagon properties (41). Quinoline hydrazones with moderate glucagon antagonist activity have been reported (42). These competitive antagonists show functional activity comparable to the binding affinity. The most potent compound in this series, **9**, binds to the human glucagon receptor with an IC_{50} of 400 nM.



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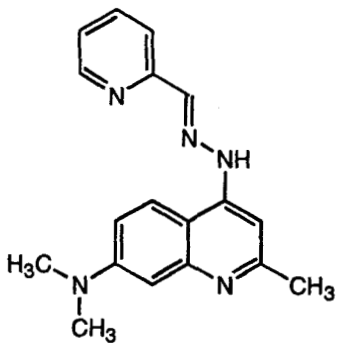
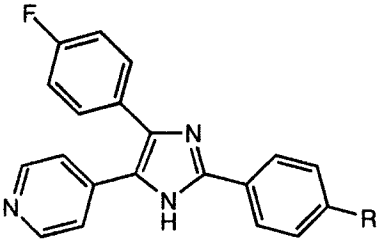


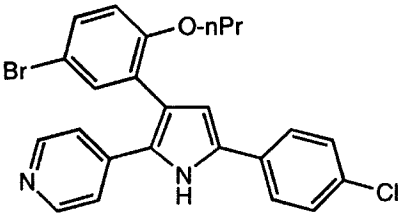
9

A series of triaryl pyrroles has recently been reported as antagonists of the glucagon receptor (43). The lead compound in the series, thioether derivative **10**, was identified from screening against the human glucagon receptor. Compound **10** is the

precursor to the known p38 kinase inhibitor **11** (SB203580) (44). Thioether **10** is only moderately selective for the glucagon receptor (IC_{50} 490 nM) compared to p38 kinase activity, IC_{50} 120 nM. Systematic modification of imidazole **10** and the related 2-pyridyl-3,5-diaryl pyrrole derivatives led to identification of potent and selective antagonists of the human glucagon receptor. The 2-propoxy-5-bromophenyl pyrrole analog **12** (L-168,049) exhibits an IC_{50} of 3.7 nM in a binding assay with the human receptor (45). Compound **12** also shows ten-fold selectivity *vs.* p38 kinase. The SAR of the series indicates that the 2,5-substitution pattern of the trisubstituted benzene, in combination with the absence of the N-H of the imidazole nucleus, contributes to improved potency and selectivity for the glucagon receptor. Compound **12** reduces cAMP stimulation by maximal glucagon and increases the rate of dissociation of glucagon from the receptor. These findings suggest that the compound is a non-competitive inhibitor of glucagon binding. Point mutations in the glucagon receptor indicate that the ligand acts in transmembrane segments 2 and 3. Receptor binding of the 2-pyridyl-3,5-diaryl pyrrole series exhibits a pronounced dependence on Mg^{2+} concentration. At physiological concentrations of 5 mM Mg^{2+}, binding affinities for the human glucagon receptor are reduced up to fifty-fold. The nature of this effect is unclear, but allosteric interaction of the divalent cation at a remote portion of the receptor may be involved (23). Compound **12** is well absorbed in rodents following oral administration. However, no effects on blood glucose are observed at 50 mg/kg *p.o.* in normal mice challenged with exogenously administered glucagon.

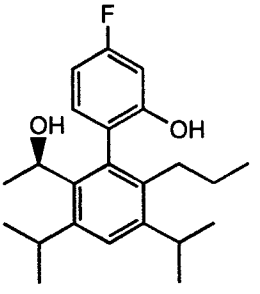


10 R = SCH_3
11 R = S(O)CH3



12 L-168,049

Substituted phenylpyridine and biphenyls have been disclosed as selective glucagon antagonists (46). The substituted biphenyl analog **13** is a potent antagonist of the human receptor with an IC_{50} of 5 nM in a binding assay. A related compound of undisclosed structure (**14**, BAY 27-9955), is reported to be in clinical development (47). Compound **14** is a competitive inhibitor of glucagon binding, acting at transmembrane segments 6 and 7. Leu^{388} in the human receptor sequence is essential for high affinity binding (48). The IC_{50} value for the inhibition of glucagon binding was 110 nM and 46 nM for the inhibition of cAMP generation by glucagon.



13

An efficacy study with **14** was conducted in normal males (47). An oral dose of 200 mg of **14** was administered 3 hours prior to conducting a glucagon challenge test. The increase in glucagon-mediated blood glucose was measured along with hepatic glucose production. During this physiologic increase in glucagon, **14** almost completely blocks the glucagon-induced increase in hepatic glucose output and reduces the elevation in blood glucose. Compound **14** is currently in Phase II clinical trials.

In summary, the only known antagonist of glucagon action in the clinic is the non-peptidyl compound **14**. Given orally, compound **14** blocks glucagon action in humans, which demonstrates the feasibility of identifying non-peptide glucagon antagonists. The next step is to determine if this approach is beneficial for the treatment of Type II diabetes.

GLUCAGON-LIKE PEPTIDE-1

Glucagon has been the target of numerous investigators in academic laboratories as well as pharmaceutical organizations. The research has provided a solid understanding of the protein and its receptor, which has facilitated the search for non-peptide ligands as potential new therapeutics. The closely related protein, glucagon-related peptide-1, was identified later as a potential molecular target for medicinal chemistry. However, based on the findings of a number of clinical and animal studies, it presents an attractive area for further investigation.

GLP-1 Receptor – GLP-1 activity is modulated through G-protein coupled receptor. A member of the incretin receptor family, the GLP-1 receptor has been characterized extensively (49,50). Cloning of the receptor confirmed its membership in the G-protein coupled receptor family (51). As pointed out previously, the GLP-1 receptor is closely related to the glucagon receptor. As such it is not surprising that the action of GLP-1 in the pancreatic islets is *via* the cAMP system (52). There is other evidence as well that GLP-1 may effect insulin secretion by raising Ca^{2+} levels (53).

The GLP-1 receptor is localized primarily in the β-cells in the pancreas, and to a lesser extent in the hypothalamus, adipose tissue and muscle (54). In a rat feeding model, repeated administration of GLP-1 (7-26) amide by intracerebroventricular injection reduces food intake and body weight, presumably through the hypothalamic receptor. By contrast, repeated *i.c.v.* administration of the GLP-1 receptor antagonist exendin (9-39) amide causes an *increase* in food intake and body weight (55). A GLP-1 receptor gene knock out model has been used to evaluate the processes controlled by this receptor (56). Findings from this animal model support the incretin activity of GLP-1 on insulin secretion and the subsequent regulation of glucose metabolism but they do not support other putative effects of GLP-1 on food and water intake (57).

GLP-1 - Peptide Agonists – There are two active forms of GLP-1 in human subjects. The predominant form is the 7-36 amide; a minor 7-37 form is also present (58). Some information is available on the amino acid residues important for GLP-1 binding to its receptor. Alanine scans indicate that His^{7}, Gly^{10}, Phe^{12}, Thr^{13}, Asp^{15}, Phe^{28} and Ile^{29} are essential for binding (59). Extending the N-terminus of the peptide, producing GLP-1 (6-37) is biologically inactive (60).

The residues in GLP-1 cited as important from the alanine scan are present in another peptide, exendin-4, a GLP-1 receptor agonist isolated from the venom of *Heloderma suspectrum* (61). Exendin-4 has a somewhat higher affinity than GLP-1 for the GLP-1 receptor. In contrast, a related protein in this venom, exendin (9-39 amide), is an antagonist to GLP-1 (62).

Systematic amino acid substitutions were done at sites on GLP-1 considered to be important in the three-dimensional structure of the protein (63). Although this work did not identify any analogs that were significantly more potent than GLP-1, a prediction was formulated for the geometry of GLP-1 contact points with the GLP-1 receptor. It was proposed that His^{7} and Tyr^{19} formed one contact point area. A second contact point area on GLP-1 was formed by Phe^{12} and Thr^{13}, and a third area was formed by Phe^{28} and Leu^{32}. Based on this model, productive binding of GLP-1 to the receptor requires the correct orientation of the C-terminal and N-terminal groups relative to each other and that the entire length of the peptide participates in generating the proper configuration.

Several programs have been initiated to develop GLP-1 or GLP-1 analogs for Type II diabetes (63,64). GLP-1 itself has been studied in clinical trials but its short half-life has hindered development. Based on its purported longer duration of action, exendin-4 is being evaluated in clinical trials (65).

Improvement in the agonist activity of GLP-1 has centered on improving its pharmacokinetic properties (66). Two distinct strategies have emerged. One approach has focused on inhibiting the activity of dipeptidyl peptidase-IV (DPP-IV). DPP-IV is the aminopeptidase responsible for the majority of GLP-1 inactivation *in vivo* (67). Not only does this enzyme inactivate GLP-1, but the product of the enzymatic action, GLP-1 (9-36), is a potential antagonist at the GLP-1 receptor (67,68).

Compounds that block DPP-IV activity in the plasma of animals have been used to test this strategy. For example, the DPP-IV inhibitor isoleucine thiazolidide improves glucose tolerance in an animal model (69). Another inhibitor, SDZ 272-070, improves glucose tolerance of obese Zucker rats and rats made insulin resistant by a high fat diet (70). A patent application has claimed cyanopyrrolidines as DPP-IV inhibitors (71). A complementary approach involves chemical modification of GLP-1 to improve metabolic stability while maintaining full agonist activity. Modification of the amine terminus of GLP-1 to reduce DPP-IV-catalyzed hydrolysis has been shown to improve antihyperglycemic activity *in vivo* (72). Similarly a "DPP-IV resistant" analog of GLP-1 has exhibited prolonged antidiabetic effects (73).

A series of GLP-1 analogs has been prepared by substitution of the Ala at the second position in the peptide (position 8 in the numbering scheme) since the N-terminal histidine is required for high affinity binding to the receptor (74). These analogs exhibited a significantly longer half-life in porcine plasma than GLP-1 (7-36), *i.e.* 159 to 197 minutes for various analogs compared to 28 minutes for GLP-1 amide. However only two of the analogs, Aib^{8} (aminoisobutyric acid) and Gly^{8} peptides, retain binding affinities for the GLP-1 receptor comparable to native GLP-1. When administered by *i.v.* infusion to pigs, these analogs demonstrated longer half-lives than GLP-1; 3.3 to 3.9 minutes compared to a GLP-1 half-life of 0.9 minutes. Although DPP-IV plasma activity was shown to be an important factor in the clearance of GLP-1, it is clear that other degradative processes are very important.

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Chapter 20. Pharmacology of Cannabinoid Receptor Agonists and Antagonists

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Introduction - Cannabinoids have been the subject of research for decades due to their unique biological effects in the CNS (1). Recently, two cannabinoid receptors (CB1 and CB2) were cloned, expressed and characterized (2-4). Since then, a number of novel cannabinoid receptor agonists and antagonists have been developed to facilitate the understanding of the physiological roles of cannabinoid receptors and their endogenous ligands. Research in this area has been reviewed (5-8), and the current review focuses only on the latest developments with cannabinoid receptor agonists/antagonists and the relevant biological effects of these compounds.

PHARMACOLOGY OF THE CANNABINOIDS

Δ^9-Tetrahydrocannabinol (Δ^9-THC, 1) is the active principle responsible for the central nervous system effects of *Cannabis sativa* in human. The brain has long been known as the principal target organ, with effects which include alterations in memory and cognition, euphoria, and sedation, and most of the findings have been reviewed (9-11). Δ^9-THC causes impairment in memory and learning, particularly short-term memory (12). In addition, peripheral effects related to bronchial constriction, immunomodulation, and inflammation have been observed (13-14). Cannabinoids also affect the cardiovascular system. Δ^9-THC can induce tachycardia, hypotension and inhibit platelet aggregation (15). However, it has been difficult to extrapolate *in vitro/in vivo* animal results to humans for many reasons, including the high dose of cannabinoids used and conflicting data obtained. A significant factor contributing to these problems is that cannabinoids at high concentrations can exert their actions through non-specific as well as receptor mediated mechanisms. Overall, evidence indicating that the peripheral effects of cannabinoids are directly mediated, and not secondary to a CNS effect, has been limited by a number of factors. These include: the lack of receptor characterization, the hydrophobic nature of Δ^9-THC, the lack of information concerning an endogenous cannabinoid ligand and, until recently, the lack of receptor subtype-selective compounds.

CANNABINOIDS AND ENDOGENOUS LIGANDS

Cannabinoids are a class of highly lipophilic benzopyrans. The best known molecule in this class is Δ^9-THC (1), the active ingredient of marijuana (16). Since Δ^9-THC shows relatively weak binding affinitiy for both the CB1 and CB2 receptors, there has been an ongoing effort to identify more potent Δ^9-THC analogs as well as its cannabimimetic analogs (17-20). CP 55,940 (2) represents one of the more potent cannabimimetic analogs that has been widely used in animal studies. The first endogenous ligand, anandamide (3), was originally isolated from mouse brain membrane (21) and was subsequently also found outside of CNS [human and rat

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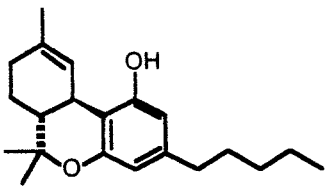
spleen, human heart and rat skin (22)]. Compound 3 binds specifically to both cannabinoid receptors and mimics the cannabinoid-induced inhibition of an electrically-stimulated twitch response in mouse vas deferens. Another endogenous cannabinoid receptor agonist, *sn*-2-arachidonyl glycerol (4), was discovered subsequently in canine small intestine and in rat brain (23-24). These endogenous compounds are generally of weak potency, having about the same affinity for the CB1 receptor as 1, and they are metabolically unstable.



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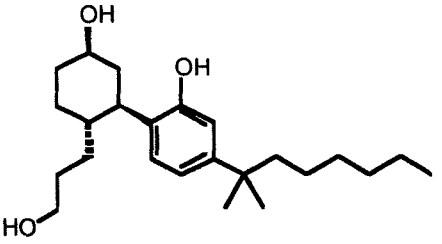

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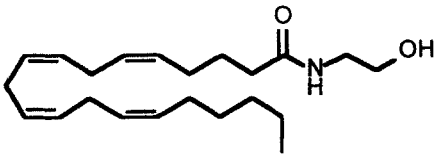

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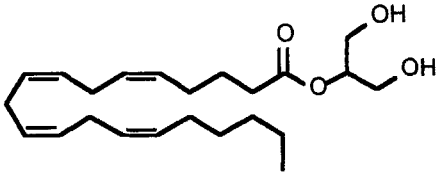
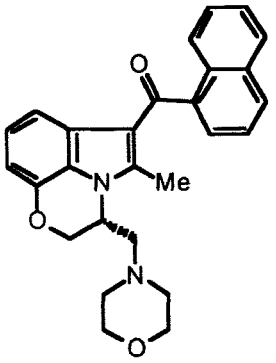


4

FUNCTIONAL ASSAYS OF RECOMBINANT RECEPTORS

CB1 and CB2 receptors are negatively coupled to adenylyl cyclase through a pertussis-toxin-sensitive Gi/o protein (25-27). More recently, compound 2 and WIN 55,212-2 (5) have been shown to activate MAP kinase in cells stably-transfected with the CB2 receptor. This effect is receptor mediated since it is also sensitive to pertussis toxin (28). There appear to be no other effector mechanisms involved in cannabinoid receptor signaling. However, there are distinct differences



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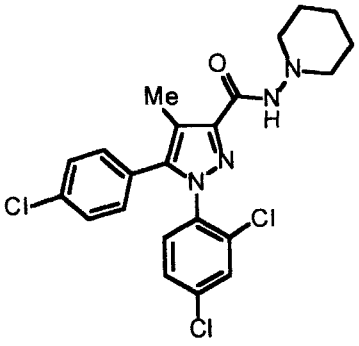


6

between the two receptors, particularly relating to effects on ion-channels. CB1, but not CB2, inhibits N- and Q-type calcium channels but activates inwardly rectifying potassium channels (26). Inhibition of N-type calcium channels decreases neurotransmitter release from the hippocampus, cortex and cerebellum (29). Several other functional assays are potentially applicable: cellular cAMP determinations, guanine nucleotide exchange, and reporter techniques such as melanophore pigment aggregation and activation of luciferase transcription. It has been reported that the CB1-selective antagonist **6** does not prevent inhibition of cAMP production mediated by the CB2 receptor (30). These assays are critical in defining the "inverse agonist" effect of antagonists. Data generated by SR 141716A (**6**) in some isolated intact tissues has suggested that this compound is an inverse agonist at the CB1 receptor. Furthermore, **6** alone inhibits basal incorporation of GTP-γS in CHO cells expressing recombinant CB1 and CB2 (31).

DISTRIBUTION OF CB RECEPTOR mRNA

The distribution of CB receptor mRNA has been examined by Northern analysis and RT-PCR. By Northern analysis, CB2 mRNA is detectable in the spleen, but not in liver, nasal epithelium, thymus, brain, lung, and kidney (4). CB1 transcripts are abundant in the brain, detectable in the testis, and are undetectable in the stomach, spleen, kidney, liver, heart, and lung (32). Thus, the majority of CB1 mRNA is localized in the brain, whereas the majority of CB2 RNA is associated with the marginal zone of the spleen, consistent with studies demonstrating specific cannabinoid ligand binding to these tissues (2,33). This simple distribution of CB1 and CB2 receptor mRNA is less clear-cut when examined by RT-PCR, a highly sensitive technique. Thus CB1 transcripts have been found in spleen, thymus, tonsils, peripheral blood leukocytes (34-35), heart, colon, stomach, liver, pancreas, lung, kidney (27), and murine uterus (36). The low level of CB1 mRNA in mouse uterus corresponds to a low-level expression of the CB1 receptor (10 fmole/mg of membrane protein), which is negatively coupled to adenylyl cyclase and induces expression of lactoferrin in an estrogen-receptor independent manner (36). Similar analyses have detected CB2 mRNA in macrophages/monocytes (4), B cells (37), and rodent mast cells (38). CB2 receptors also mediate agonist dependent proliferation of human B cells and degranulation of rodent mast cells (37, 38). Therefore, low level expression of CB1 and CB2 receptor mRNA does not necessarily indicate an artifactual or non-physiological role for cannabinoid receptors in target tissues or cell lines.

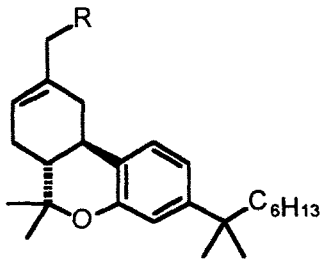
RT-PCR has been utilized to examine the distribution of CB1 and CB2 receptor mRNA in various Wistar rat tissues and RBL-2H3 cells, a rat mast cell line that expresses CB2 mRNA and releases serotonin upon incubation with cannabinoid agonists (39). The results qualitatively confirm that CB1 and CB2 mRNA are predominantly found in the brain and spleen, respectively. CB2 mRNA is also found in the brain and cerebellum, but apparently at lower levels than CB1 transcripts. In addition, CB2 transcripts are found in several peripheral tissues at levels that appear comparable to the levels found in RBL-2H3 cells. Although contaminating blood and lymphocytes may contribute to the presence of CB2 mRNA in various tissues, it is interesting to note a potential correlation between the presence of CB2 mRNA in aorta, heart and kidney. Furthermore, a transient pressor effect of *iv* anandamide and Δ^9-THC mediated by non-CB1 receptors (resistant to **6**) by a mechanism independent of sympathetic innervation has been reported (40).

ALTERATIONS OF CB RECEPTOR mRNA IN DISEASE

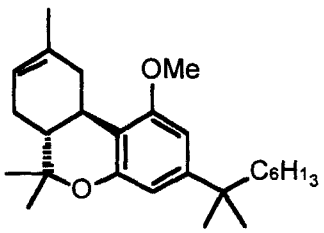
There is a paucity of studies linking the expression of cannabinoid receptors or specific endogenous ligands to the etiology of diseases. Behavioral responses such as movement control have been suggested to be a result of decreased CB1 receptor expression in Huntington's disease (41). Blood anandamide levels are being investigated to determine its role in early disease progression. Agonists and antagonists to the CB2 receptor have not been fully used to study disease pathogenesis.

DESIGN OF RECEPTOR LIGANDS

Agonists – One approach adopted to identify more potent and/or selective CB agonists has been continued investigation of classical cannabinoid chemistry. It was found that a phenolic hydroxyl at C1 is not essential for cannabinoid activity. Replacement of the phenolic hydroxyl group either with hydrogen or with a methoxy group resulted in compounds with greater potency and selectivity for the CB2 receptors (42,43). 1-Deoxy-HU210 (**7**) displayed a high affinity for the CB2 receptor, with a Ki of 0.032 nM and selectivity for the CB2 receptor increased by one order of magnitude compared with HU210 (**8**). L-759,633 (**9**) and L-759,656 (**10**), on the other hand, showed excellent selectivity for the CB2 receptor, with CB1/CB2 ratio of 793 and >1000, respectively, while maintaining good CB2 receptor affinity (Ki = 20 and 19 nM, respectively). Further modification of the cannabinoid template by replacing the pyran oxygen with nitrogen led to a new class of molecules, whose structures are similar to levonantradol. L-759,632 (**11**) showed somewhat weaker activity for the CB2 receptor (Ki = 55 nM), but the selectivity for this receptor over CB1 was still significant (500-fold). (44)



7 R = H
8 R = OH



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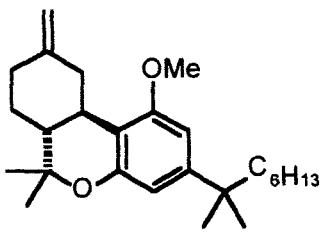

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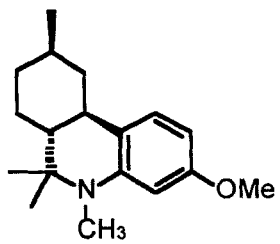
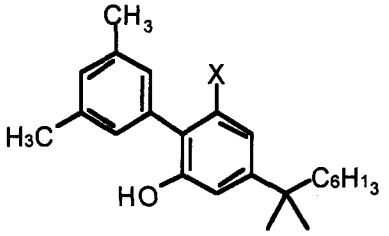


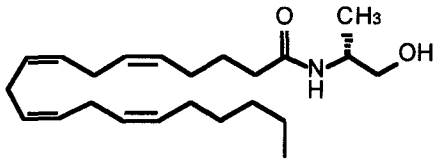
11

Other non-tricyclic analogs of cannabinoids (e.g., biphenyls) have been prepared (45). The most potent compound for the CB2 receptor in the biphenyl series is the dihydroxybiphenyl (**12**). Compound **12** displayed low nanomolar potency (Ki = 2 nM) for the CB2 receptor and exhibited a 40-fold selectivity over the CB1 receptor. Interestingly, removal of one of the two hydroxyl groups led to compound **13**, with increased affinity for CB1 receptor as well as high affinity for CB2 receptor (Ki = 4 and 13 nM for CB2 and CB1, respectively).



12 X = OH
13 X = H

A series of anandamide analogs has been reported and some of these analogs were found to be more potent and more stable than the endogenous ligands **3** and **4** (46-50). (R)-Methanandamide (**14**) showed an improved metabolic stability. Unlike cannabinoid analogs **9** and **10**, this compound showed a binding preference for the CB1 receptor with Ki values of 20 nM and 815 nM for CB1 and CB2 receptors, respectively (51). The fatty acid amides **15**, **16** and **17**, on the other hand, showed reversed selectivity in favor of the CB2 receptor (39,52,53). Compound **15** is orally active in reducing edema formation and inflammatory hyperalgesia by down modulating mast cell activation (54) and is in clinical development for treatment of inflammation (55).



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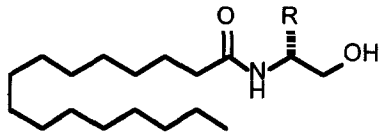
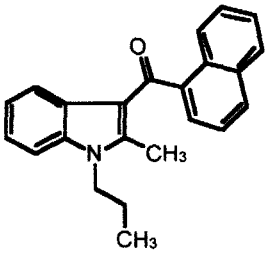


15 R = H
16 R = Ph
17 R = CO_2H

The design of new aminoalkylindoles has continued to be the focus of several research groups in recent years (56-58). In addition to the previously discovered WIN 55212-2 (**5**) (Ki = 1.9 nM and 0.3 nM for CB1 and CB2, respectively), less conformationally restricted analogs such as **18** and **19** showed improved selectivity for the CB2 receptor. Compound **18** displayed receptor binding affinity with Ki values of 14 nM for CB2 and 383 nM for CB1 (59). The morpholine-containing analog **19** showed a superior CB2 binding (Ki = 1.8 nM) and selectivity (CB1/CB2 ratio >500) profile to that of **18**. Transposing the 1 and 3 substituents on the indole ring also provided potent, CB2 selective compounds. L-759,787 (**20**) and L-768,242 (**21**) exhibited high affinity for the human CB2 receptor, with Ki values of 8.5 nM and 12 nM, respectively. The selectivity for CB2 over CB1 is greater than 100-fold for both compounds.



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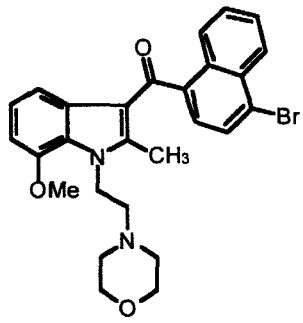

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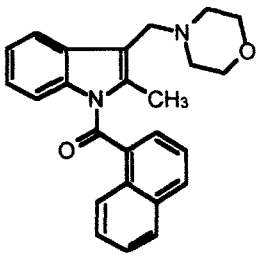

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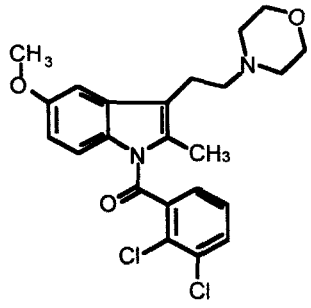
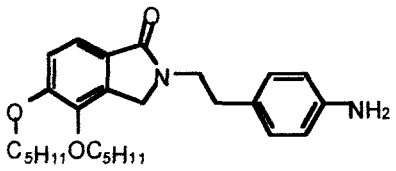


21

Isoindolinone and quinoline derivatives 22 and 23 have been recently reported as new series of selective cannabinoid agents (60,61). Compound 22 is one of the most potent, CB2-selective agonists reported to date. It showed IC_{50} values of 0.11 nM and 330 nM for CB2 and CB1, respectively. Compound 23 showed *in vivo* activity, with an ED_{50} of 0.04 mg/kg (po) in the carrageenan-induced edema model in ddY mice.



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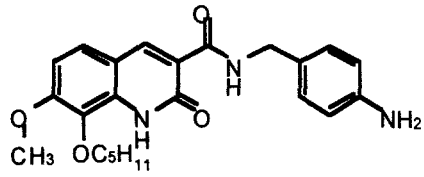
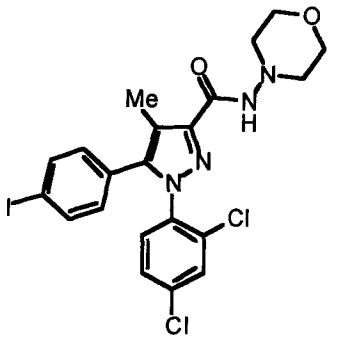


23

Antagonists - Since the first potent, CB1-selective antagonist, SR 141716 (6), was disclosed in 1994 (62), few compounds have been reported as CB1 selective antagonist in this class except the iodinated analog 24. This compound has been used as a radio-labelled CB1 ligand for SPECT (single photon emmission computed tomography) imaging studies (63), and its fluorinated derivative 25 is claimed in a patent for PET (positron emission tomography) imaging studies (64). Compound 6 has been studied in several biological systems *in vivo*. The compound was shown to attentuate methoxamine-induced tone of rat isolated mesenteric artery induced by 3, 2, and 8 but not by palmitoylethanolamide and 5 (65). The inhibitory effect of

cannabinoids on acetylcholine output in medial-prefrontal cortex and hippocampus was blocked by **6** (3 mg/kg), while the compound alone caused an increase in basal acetycholine release (66). Another compound, LY-320135 (**26**), has emerged from a series of arylbenzofurans as potent CB1-selective antagonist (67). In binding assays, **26** exhibited Ki values of 141 nM and 14900 nM for CB1 and CB2 receptors, respectively (68). *In vivo*, this compound at 20 mg/kg (ip) completely reversed anandamide-induced severe depression in mice.

More recently, a CB2 selective antagonist, SR 144528 (**27**), has also been reported from the diarylpyrazole series (69,70). Through placement of a benzyl group and a fenchylamide substituent at the 1 and 3 positions, respectively, of the pyrazole ring, the potency and selectivity profile of resulting compound **27** is completely reversed from that of **6**. Thus, **27** displayed a subnanomolar potency for the CB2 receptor, with greater than 700-fold selectivity over the CB1 receptor (Ki = 0.6 and 437 nM for CB2 and CB1, respectively). This compound is orally active and functionally antagonizes the effects of cannabinoid agonists on adenylyl cyclase and MAP kinase in CHO cells expressing CB2 receptors, but has no effect on CB1 receptors in the same cell lines. *In vivo* administration of SR 1444528 competitively displaces the *ex vivo* CP 55,940 binding to mouse spleen membranes, without affecting binding to membranes from brain tissue.



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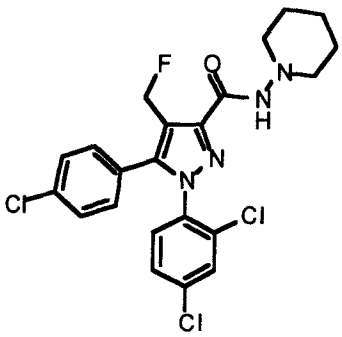

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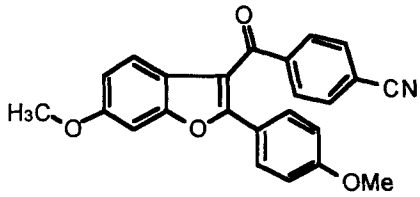

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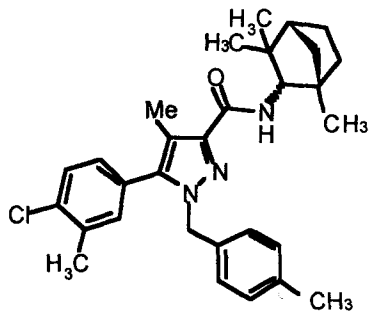
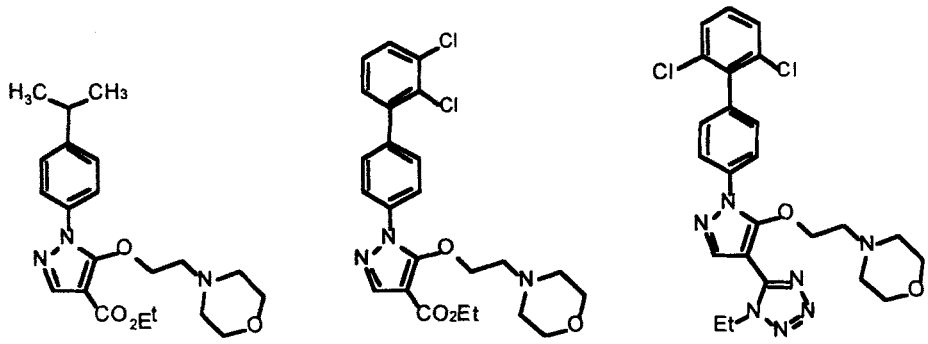


27

Another series of pyrazole antagonists was disclosed recently (71,72). Deconvolution of a 10-component sublibrary (mixture IC_{50} = 2.7 uM for CB2 receptor) led to identification of **28** as a submicromolar CB2 antagonist, with a Ki value of 280

nM. Molecular overlay of **28** with **13** suggested that the isopropyl group in **28** may be replaced with a substituted phenyl group to provide compounds with increased potency. Indeed, the 2,3-dichlorophenyl analog **29** showed an order of magnitude improved potency (Ki = 30 nM for the CB2 receptor) relative to **28**. However, the ester function was rapidly hydrolyzed to the corresponding acid *in vivo* in rat. To circumvent this problem, the metabolically stable bioisostere **30** was subsequently developed. Though **30** was less potent (Ki = 835 nM for the CB2 receptor) than **29**, it displayed greater than 100-fold selectivity for CB2 receptor.



28 **29** **30**

Conclusion - Recent advances in cannabinoid research have reached a new plateau, where much of the phenomenology has been translated into molecular terms. Molecular biology has provided the much-needed tools to define the receptor subtypes and their distribution in tissues and even in disease states. The availability of tool compounds, particularly the potent and selective cannabinoid receptor antagonists, will validate the relative importance of the two receptor subtypes and increase our understanding of signaling mechanisms. While excellent selectivity and *in vitro* potency has been achieved, the compounds discovered to date have poor pharmacokinetics profile and/or poor *in vivo* efficacy. Overlapping receptor expression in tissues also contributes to the complexity of identifying a therapeutic indication. Antagonists for either receptors may suffer from the " inverse agonist" effect may have limited clinical utility. It is hoped that additional pharmacological studies employing improved tool compounds discussed here will better define attractive clinical indications.

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Chapter 21. Non-peptide Somatostatin Receptor Ligands

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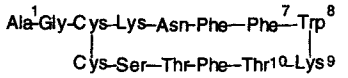
Introduction – Somatostatin (somatotropin release-inhibiting factor, SRIF) was originally isolated from mammalian hypothalamus as a potent inhibitor of pituitary growth hormone (GH) secretion (1-3). It occurs in two biologically active forms, a tetradecapeptide somatostatin-14 (1) and an N-terminal elongated 28-residue peptide somatostatin-28. Cortistatin, possibly a third endogenous ligand, has been identified (4, 5). Somatostatin is widely distributed throughout the endocrine and central nervous systems and peripheral tissues. It affects diverse biological processes such as hormone secretion, neurotransmission, cell proliferation, smooth muscle contraction, nutrient absorption, and inflammation (6-13). A number of reviews on somatostatin receptors and peptide agonists have appeared (14-17). This article will focus on advances towards discovering nonpeptide somatostatin ligands since 1990.



1: somatostatin-14

SOMATOSTATIN RECEPTORS AND PEPTIDE LIGANDS

Tremendous progress has been made in somatostatin research since it was last reviewed in this series (18, 19). It is now known that somatostatin elicits its effects through a family of G-protein-coupled receptors (GPCR) with seven transmembrane (TM) domains (20-,25) that are encoded by nonallelic genes located on separate chromosomes (26). Five receptor subtypes termed sst_{1-5} have been identified (27). Only sst_2 has two isoforms sst_{2A} and the C-terminal shorter sst_{2B}, which have indistinguishable pharmacological profiles (15, 28). Sequence homology is 39-57% among the five subtypes, and each subtype is highly conserved across different species. Somatostatin-14 and somatostatin-28 bind to all five receptor subtypes with high affinity (26), with the latter showing greater affinity to sst_5 (23). Based on extensive mutagenesis and analogies to other GPCRs, ligand-binding domains are proposed to involve TMs III-VII, with potential contributions from extracellular loop 2 (14).

Peptide Somatostatin Agonists – Somatostatin is a highly flexible cyclic peptide with a very short biological half-life. Much of the earlier work in somatostatin agonist research was centered on designing smaller cyclic peptides with a more defined conformation and longer duration of action (29). Replacing Trp^8 with D-Trp led to potency enhancement in somatostatin (30) and its analogs. Extensive efforts in the late seventies and eighties led to the design and synthesis of longer acting peptides. Most well known are the cyclic hexapeptide seglitide (MK-678, 2) (31) and cyclic octapeptides octreotide (SMS 201-995, 3) (32), lanreotide (BIM 23014, 4) (33), and vapreotide (RC-160, 5) (34). 3 is used clinically for the treatment of acromegaly and endocrine gastrointestinal tumors. 4 and 5 are currently undergoing clinical trials in Europe. These agonists were discovered with a rat pituitary GH release inhibition

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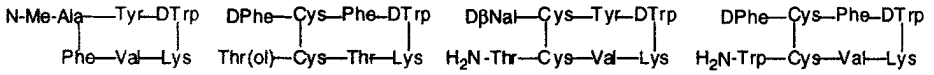
assay that is mediated through sst_2. Consequently, they are generally selective against the sst_2 receptor although they also bind to sst_3 and sst_5, but poorly to sst_1 or sst_4. The five subtypes are therefore grouped into two subfamilies, $sst_{2,3,5}$ and $sst_{1,4}$. Numerous cyclic, bicyclic as well as linear peptides have been reported (15, 28, 35) some of which were claimed to be selective toward sst_1 (36, 37), sst_3 (15) and sst_5 (38, 39). There were controversies on the selectivity of the earlier analogs because of different assay conditions among different labs (40, 41). The tetrapeptide sequence of Phe-Trp-Lys-Thr which is present in a type II' β-turn (42-,47) is highly conserved among them with Trp and Lys critical for bioactivity. Extensive molecular modeling and NMR studies of somatostatin and its various analogs have given rise to pharmacophore models (48), which may facilitate the design of more potent or selective new analogs (49).



2: seglitide **3**: octreotide **4**: lanreotide **5**:vapreotide

Peptide Somatostatin Antagonists – Selective somatostatin antagonists have long been sought as biological tools and therapeutic agents. A linear octapeptide (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH_2) was reported to be a sst_5 selective antagonist with IC_{50} of 100nM (50). By inverting the chirality of some somatostatin agonists at positions 5 and 6 (somatostatin numbering, D^5,L^6 to L^5,D^6), a highly potent antagonist (Ac-Npa^5-c(DCys^6-Tyr-DTrp-Lys-Thr-Cys)-DTyr-NH_2) was achieved (51). It inhibited somatostatin-mediated inhibition of cAMP accumulation in rat somatotroph GH4C1 cells, cells transfected with sst_2 and sst_5, and somatostatin-stimulated growth of yeast cells expressing the sst_2. Additional studies resulted in a sst_2 selective antagonist (H-Nal-c(DCys-Pal-DTrp-Lys-Val-Cys)Nal-NH_2) (52). It reversed somatostatin-induced inhibition of pentagastrin-stimulated gastric acid secretion in conscious, chronic gastric fistula equipped rats (53). By screening a hexapeptide combinatorial library, an all D-hexapeptide(Ac-DHis-DPhe-DIle-DArg-DTrp-DPhe-NH_2) was identified as an antagonist of somatostatin on sst_2 (54). It binds to sst_2 with an affinity of 170 nM, and induced GH release when given alone (50 μg, i.v.) to anesthetized rats with or without pretreatment with a long-acting somatostatin agonist.

Physiological Functions Of Somatostatin Receptors – Investigations utilizing subtype selective somatostatin analogs in both in vivo and in vitro experiments have facilitated the understanding of the biological functions of sst. Several studies have suggested sst_2 mediates the inhibition of glucagon release from the pancreas (55), gastrin and acid secretion (53, 56, 57), and prolactin (PRL) secretion (58), whereas sst_5 regulates the release of insulin (55). The secretion of GH and thyroid-stimulating hormone (TSH) are mediated through both sst_2 and sst_5 (58). The functions of other receptor subtypes remain unclear because of the lack of $sst_{1,3,4}$ selective ligands. The identification of a number of nonpeptide potent and subtype selective agonists (vide infra) should resolve this issue in the near future. sst_2 knockout mice have been generated (59). The knockout mice appeared to develop and grow normally, suggesting a nonessential role for sst_2 in mice. Somatostatin or sst_2 selective agonists had no effect on gastric acid release in the sst_2 knockout mice, indicating sst_2 mediates gastric acid secretion (57). Selective antibodies against all five human somatostatin receptor subtypes have been reported (60), and studies should shed more light on distribution and functions of the receptor subtypes. The antiproliferative activities of somatostatin are mediated indirectly through the inhibition of growth factor release, and directly via its receptors inducing growth arrest and apoptosis (10, 14, 61). A somatostatin analog TT-232 (D-Phe-(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH_2), which does not inhibit octreotide binding to

brain/pituitary membranes, was reported to have strong in vitro and in vivo antitumor activity (62). Somatostatin is also a modulator of the immune system (63, 64).

Therapeutic Targets – With the diverse biological functions of somatostatin, there is substantial potential for the therapeutic application for subtype selective agonists/antagonists (15, 65-67). Octreotide has been used experimentally for the treatment of a number of diseases (68, 69) including diabetes (70) and diabetic retinopathy, nephropathy, and neuropathy (71, 72). Murine model studies suggest that systemic inhibition of GH or IGF-I, or both, may have therapeutic potential in preventing some forms of retinopathy (73). Octreotide has also been used for the treatment of pain (74, 75), migraine (76), restenosis and chromic rejection (77), and for the treatment and diagnosis of cancer (78). A recent study indicated sst_5 selective agonists may be more effective in the treatment of GH- or PRL-secreting adenomas (79). Other potential targets for somatostatin analogs include rheumatoid arthritis (80) and Alzheimer's disease (81). A sst_2 antagonist increases GH secretion and may serve as an alternative to GH replacement therapy (54).

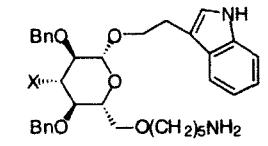
NON-PEPTIDE SOMATOSTATIN LIGANDS

While tremendous progress has been made in the identification of peptide antagonists, reports of peptide agonists have been limited (82). The discovery of morphine binding to the enkephalin receptors provided experimental evidence that peptide backbones may not be necessary for receptor binding. This, in turn, provided the theoretical basis for designing small molecules to mimic the functions of peptides. Work with peptide somatostatin analogs had demonstrated that the peptide backbone serves as a scaffold for the side chain orientations and is not important itself for binding to the receptor (83). Structural information from small cyclic peptides set the stage for the discovery of nonpeptide somatostatin mimetics.

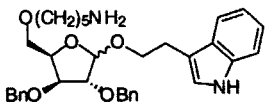
Initial efforts in nonpeptide research concentrated on structure-based design to mimic the β-turn presented in the cyclic peptide. These efforts generated several structures with modest binding affinity to somatostatin receptors. The results validated the important concept of structure-based ligand design (84), however, the ligands generated from these approaches were quite weak. Furthermore, the lack of stereospecific binding was troublesome and they were unattractive as leads for further studies. Nevertheless, these efforts demonstrated that it is possible to design small molecule peptidomimetics for somatostatin. The second approach of "directed-screening" was extremely successful in the identification of leads for sst_2 and sst_4 selective compounds. Combinatorial libraries based on a sst_2 lead also yielded other subtype selective compounds. The third approach of screening random combinatorial libraries or targeted libraries generated subtype selective compounds for a variety of receptors.

Non-selective Somatostatin Ligands – Nonpeptide somatostatin mimetics using sugars as a scaffold have been reported (85-88). The compounds were designed based on the conformational analysis of somatostatin analogs and molecular modeling studies. The objective was to direct the Trp and Lys side chains in approximate β-turn orientations to mimic their placements in the cyclic peptides. Compounds **6** and **7** displaced the somatostatin analog [^{125}I]-CGP 23996 from a mixture of somatostatin receptors on AtT-20 cells with IC_{50}'s of 10 and 1.3 μM, respectively. Functional studies (GH release from cultured rat anterior pituitary cells with and without somatostatin) suggest **7** is a partial agonist of somatostatin. Inspired by the pyranose design, a

number of structures based on other scaffolds were prepared. A xylofuranose **8** displaced [^{125}I]-octreotide with an IC_{50} of 23 μM (89). In the same assay conditions, **7** showed an IC_{50} of 16 μM, suggesting that the 5- or 6- membered sugar templates afford similar results. A benzodiazepine-based compound **9** (IC_{50} = 7 μM) is 3-fold more potent than **8**, which can be attributed to different conformation and rotational freedom between the two series (90). A number of aza-sugar based compounds were reported to be mimetics of somatostatin (91). Systematic studies were carried out on the effect of ring size (five to seven) and on different substitution patterns as well as ring restrictions. All these variations gave compounds that displaced [^{125}I]-Tyr^{11}-somatostatin from rat cerebral cortex with IC_{50} values between 10 and 15 μM. Representative compounds include **10** (14 μM) and **11** (12 μM). In a recent report, a proline based spirolactam scaffold was designed as a novel β-turn peptidomimetic. **12** and its various isomers showed binding affinities of 11-22 μM (92).



6: X = H
7: X = OBn



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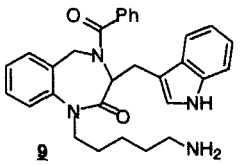

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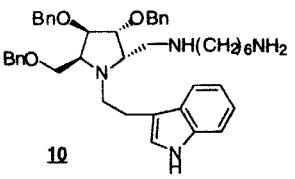

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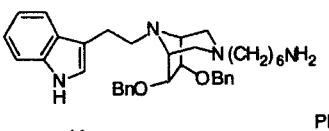

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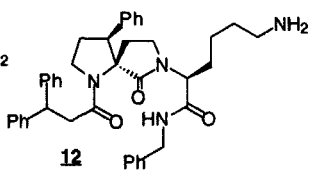
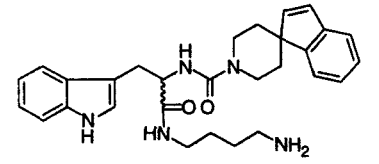


12

sst_2 Selective Somatostatin Agonists – A series of potent and selective small molecule human sst_2 agonists has been reported (93-95). Directed screening was used to discover the original lead **13** (IC_{50} = 615 nM, sst_2) from which all the sst_2 selective compounds and two other receptor selective (sst_1, sst_3) compounds were developed. A pharmacophore model of a basic amine (for Lys^9), an indole (for Trp^8) and another lipophilic group was proposed based on the cyclic peptide work, and sample collection searching identified **13**. It originated from a new approach, namely the derivatization of "privileged structures" (96), for receptor ligand identification. The method had been successfully used in the development of potent growth hormone secretagogues (97). Extensive SAR studies were carried out with this highly modular lead using both traditional medicinal chemistry and combinatorial approaches. By constraining the amino side chain with cyclohexane, the first potent and selective low molecular weight sst_2 agonist **14** was identified (93). It binds to human sst_2 with high selectivity (IC_{50} (nM): sst_2: 1.6; sst_1: 1740; sst_3 2950; sst_4: 2000; and sst_5: 4470). **14** showed full agonism on sst_2 in the inhibition of forskolin-stimulated accumulation of cAMP (cAMP assay) with an IC_{50} of 2 nM, and it also inhibited GH release in the rat pituitary assay with IC_{50} of 6 nM.

Incorporation of (2*R*,3*S*)-β-Me-Trp (98), LysOBu^t, and a benzimidazolonyl-piperidine into the design resulted in **15**, which binds to sst_2 with IC_{50} of 0.01 nM (vs. 0.04 nM for somatostatin) (94). It was at least 3000 fold selective over the other four receptor subtypes (IC_{50} (nM): sst_1: 2392; sst_3 31; sst_4: 81; and sst_5: 163). It is a full agonist based on the cAMP assay (IC_{50} = 0.1 nM) and the GH release inhibition assay (IC_{50} =0.05 nM), being more potent than somatostatin itself in both assays. **15** is a potent

inhibitor of glucose-stimulated release of glucagon from isolated mouse pancreatic islets with an IC_{50} of 0.05 nM. It also inhibited the release of insulin in the same system with an IC_{50} of 12 nM (vs. 0.3 nM for somatostatin), possibly reflecting its 163 nM affinity to sst_5. Intravenous infusion of **23** to rats at 50 μg/kg·h caused a rapid and sustained reduction in GH to basal levels.



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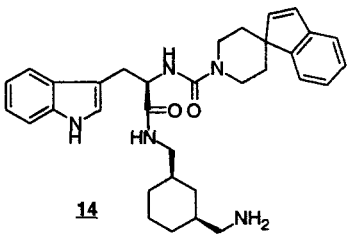

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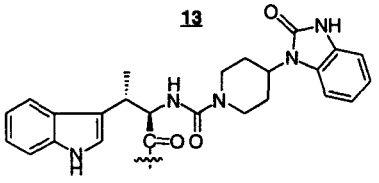

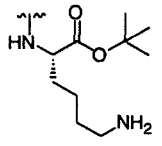

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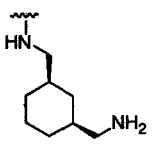

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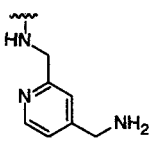

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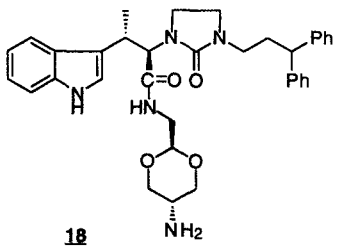
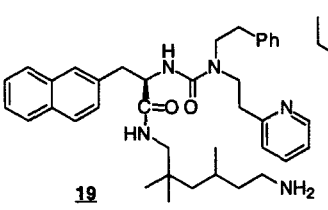


18

An exceptionally selective sst_2 agonist **16** (IC_{50} (nM), sst_2: 0.05; sst_1: 2760; sst_3: 729; sst_4: 310; sst_5: 4260) was discovered by deconvolution of a combinatorial mixture (95). **16** is a full agonist with an IC_{50} of 0.05 nM in the cAMP assay, 0.025 nM in inhibiting GH release from rat pituitary cells, and 0.1 nM in inhibiting arginine-stimulated secretion of glucagon from mouse pancreatic islet cells. Its higher selectivity against sst_5 reflected its decreased inhibition of insulin release with an IC_{50} of 100 nM compared to 12 nM for that of **15**, providing additional evidence that insulin secretion is mainly regulated by sst_5. A closely related analog **17** with similar potency and selectivity profiles (IC_{50} (nM), sst_2: 0.06; sst_1: 4560; sst_3: 391; sst_4: 4600, sst_5: 1120) as **16** was recently disclosed (99).

Backbone cyclization of these urea-based leads resulted in the first reported orally bioavailable somatostatin agonists typified by the cyclic urea **18** with a K_i of 8.5 nM on sst_2 (100). It had good selectivity for sst_2 and an oral bioavailability of 64% in rats.



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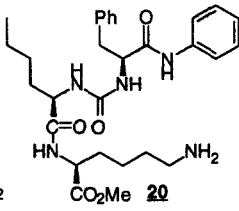

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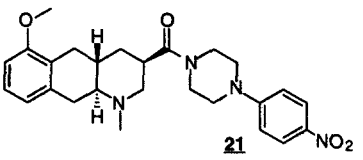
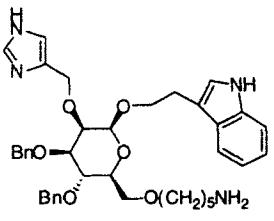


21

sst_1 & sst_3 Selective Somatostatin Ligands –The identification of sst_1 and sst_3 selective agonists from a structurally identical but expanded combinatorial library has been reported (95). **19** is highly potent and selective towards sst_1 (IC_{50} (nM), sst_1: 1.4; sst_2: 1875; sst_3: 2240; sst_4: 170; sst_5: 3600), and demonstrated full agonist activity in

the cAMP assay with an IC_{50} of 3 nM. **20** is a sst_3 selective compound (IC_{50} (nM), sst_1: 1255; sst_2: >10000; sst_3: 24; sst_4: 8650; sst_5: 1200), and was found to be a partial agonist with an IC_{50} of 18 nM for inhibition of forskolin-stimulated cAMP production. Neither compound (**19**, **20**) inhibited the release of GH, glucagon or insulin.

A patent application describing a series of novel benzo[g]quinoline derivatives as sst_1 selective antagonists recently appeared (101). The specifically claimed compound **21** showed an IC_{50} of 0.8 nM and 19 nM on rat and human sst_1, respectively, without significant activity for a wide range of neurotransmitter receptors. These antagonists are reported to be active in three described behavioral tests, which are considered predictive of uses in the treatment of anxiety, depression and bipolar disorders.



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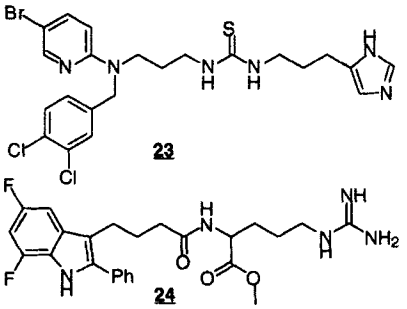
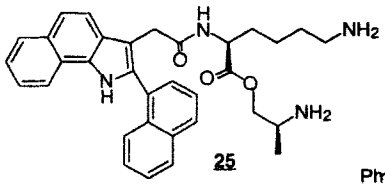


23

24

sst_4 Selective Somatostatin Ligands – Recently, it was reported that by incorporation of an imidazole, the sugar derivative **22** is a modestly active and selective sst_4 ligand (IC_{50} (μM), sst_4: 0.1; sst_1: 0.3; sst_2: 0.8; sst_3: 0.9; sst_5 >10) (88). sst_4 Selective agonists in a series of thioureas have been reported (102-104). Compound **23** (NNC 26-9100) is potent and selective for sst_4 (IC_{50} (nM): sst_1: 1800; sst_2: 621; sst_3: 1400; sst_4: 6; sst_5: 1900). It is a full agonist as it inhibited forskolin-induced accumulation of cAMP in baby hamster kidney cells, expressing $hsst_4$, with an EC_{50} of 2 nM. The paper indicated studies are in progress for the treatment of glaucoma, but no data were given. An even more potent and selective sst_4 agonist **24** (IC_{50} (nM): sst_1: 199; sst_2: 4720; sst_3: 1280; sst_4: 0.7; sst_5: 3880), was discovered from screening of a 2-arylindole combinatorial library (95). It is a full sst_4 agonist in the cAMP functional assay, and it did not inhibit secretion of GH, insulin, or glucagon.



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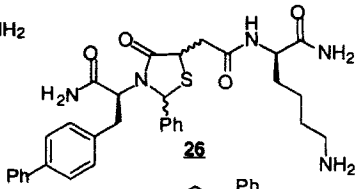
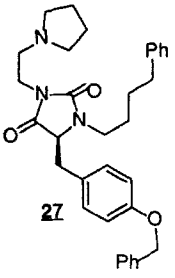
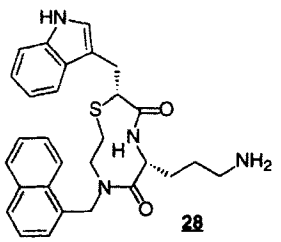


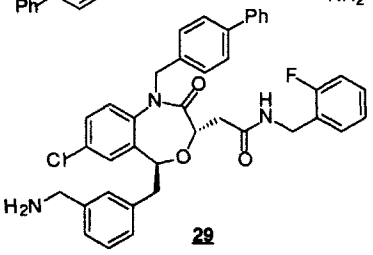
26



27



28



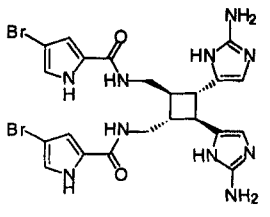
29

sst$_5$ Selective Somatostatin Ligands – A potent and modestly selective sst$_5$ agonist **25** (IC_{50} (nM), sst$_5$: 0.4; sst$_1$: 3.3; sst$_2$: 52; sst$_3$: 64; sst$_5$: 82) was also identified through the screening of a 2-arylindole combinatorial library (95). It inhibited insulin secretion from pancreatic islets (IC_{50} = 0.3 nM) and did not effect glucagon release (IC_{50} > 30 nM), supporting earlier conclusions made with sst$_5$ selective peptides. It inhibited GH release in the rat pituitary assay with an IC_{50} of 3 nM, suggesting sst$_5$, together with sst$_2$, regulates GH release.

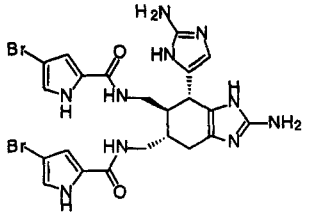
A series of trisubstituted hydantoins has been reported to have submicromolar affinities for human sst$_5$ (**26**, IC_{50} = 0.3 μM) with no more than 50% inhibition on sst$_2$ at 1 μM (105). These hydantoins were designed based on a thiazolidinone lead AF 15831 (**27**, IC_{50} = 1.4 μM, sst$_5$) obtained from general screening of combinatorial libraries (106).

A strategy for receptor ligand identification by preparing and screening focused, macrocyclic libraries mimicking β-turns has been reported (107). Screening of this library yielded **28** (IC_{50} (nM), sst$_5$: 87; sst$_1$: 501; sst$_2$: 1585; sst$_3$: 3090; sst$_4$: 1047). A number of very closely related compounds also showed submicromolar affinity to other somatostatin receptor subtypes. No functional data were presented.

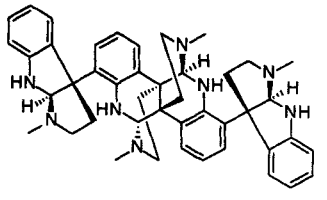
A series of 4,1-benzoxazepine somatostatin agonists, structurally similar to **9**, was reported in a patent application (108). The reported data indicates the benzoxazepines bind to the subclass of sst$_{2,3,5}$ better than to sst$_{1,4}$. Compound **29** (IC_{50} (nM), sst$_1$: >1000; sst$_2$: 60; sst$_3$: 80; sst$_4$: 300; sst$_5$: 0.3) showed selectivity toward sst$_5$, and represents one of the most potent and selective nonpeptide sst$_5$ agonists reported to date. It also inhibited GH secretion from rat pituitary with an IC_{50} of 10 nM, providing further evidence that sst$_5$ plays a part in GH regulation.



30: Sceptrine



31: Ageliferine



32: Psycholeine

Natural Products As Somatostatin Receptor Ligands – Sceptrine (**30**), and ageliferine (**31**), isolated from *Xestospongia* sp. and *Agelas novaecaledoniae* exhibited binding affinities to the somatostatin receptor with IC_{50}'s of 0.27 μM and 2.2 μM respectively (109). Psycholeine (**32**), a natural alkaloid from *Psychotria oleoide*, acts as a weak antagonist of somatostatin (IC_{50} = 10 μM) (110).

Conclusion – Somatostatin receptor modulation will continue to be the focus of research since it provides opportunities for intervention in a number of therapeutic targets. Clinical data derived from octreotide in the treatment of a variety of diseases reiterates the validity of this approach. The identification of small molecules as potent and selective agonists/antagonists will facilitate target validation in animal models. It is anticipated that medicinal chemistry efforts will continue to develop potent, selective and orally bioavailable agents for each of the receptor subtypes.

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SECTION V. TOPICS IN BIOLOGY

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Chapter 22. Cytokines in T Cell Maturation

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Introduction- Naïve T cells can differentiate along Th1 or Th2 pathways after stimulation with antigen depending on factors such as the nature of the antigen, antigen presenting cells, and cytokine milieu (1). The Th1 cells produce proinflammatory cytokines such as IL-2, IFNγ and TNF and can have cytotoxic functions. In addition, they are important in cell-mediated inflammatory responses, such as delayed type hypersensitivity (DTH) reactions. The Th2 cells produce cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 that are important for class switching in antibody responses and in mediating allergic reactions. Cytokines produced by Th1 can modulate the development and functions of Th2 and vice versa.

IL-12 was originally described as an NK cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF) (2). IL-18 was discovered as an inducer of IFNγ production (3). It is now clear that IL-12 and IL-18 share three inter-related functional properties and can synergize in: (i) induction of IFNγ production by Th1 and NK cells; (ii) functional development of Th1 and NK cells and; (iii) biasing the immune responses towards Th1 and NK cell-dependent proinflammatory responses (4,5). Recent studies show that IL-12 and IL-18 can also synergize to induce IFNγ production in macrophages; however, these cytokines are ineffective alone (6). This review will summarize the known properties of IL-12 and IL-18, their roles in various inflammatory diseases, and therapeutic strategies available for neutralizing these cytokines for the treatment of inflammatory diseases.

IL-12 AND IL-18 RECEPTOR-MEDIATED SIGNALLING

IL-12 - Bioactive IL-12 is a 70 kDa disulfide-linked heterodimer of p40 and p35 subunits (2). These subunits share no significant homology and are encoded by genes on different chromosomes. The p35 subunit is constitutively expressed on many cells, but p40 expression is restricted to IL-12 producing cells and is inducible. The p35 subunit cannot be released from the cells without forming heterodimers with p40, but the p40 subunit can be released alone from cells without pairing with p35 and can exist as monomers or homodimers (2,5). IL-12p40 homodimers can bind to the IL-12 receptor without activating it and thereby serve as IL-12 antagonists, at least in mice (5,7).

There are two subunits of the IL-12 receptor, β1 and β2. Several combinations of these have been described and they each have different affinities for IL-12 (Kd: 5-20 pM, 50-200 pM, 2-6 nM) on T cells (5). The β2 subunit is thought to be involved in the intracellular signaling. Naïve T cells express the β1 subunit alone, and upon activation and differentiation into Th1 cells, express the β2 subunit in addition (8,9). Thus the β2 subunit serves as marker for Th1 cells. Binding of the IL-12 heterodimer to the IL-12R leads to activation and phosphorylation of two non-receptor tyrosine kinases in the Janus family, JAK2 and TYK2 (10). Activation of these kinases results in the recruitment and phosphorylation of two cytosolic

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transcription proteins belonging to the Signal Transducer and Activator of Transcription (STAT) family, STAT4 (mainly) and STAT3 (11,12). The activated STAT proteins can dimerize via their SH2 domains, and translocate to the nucleus where they initiate the transcription of other cytokines such as IFNγ. STAT-4 knockout mice have defective IL-12 signaling and impaired Th1 responses similar to IL-12 knockout mice (13-15). Hence, STAT4 is critical for IL-12 signaling, and is a potential target for the discovery of drugs to inhibit the effects of IL-12. However, IFNα/β also can activate STAT4, at least in humans, and it is not clear if STAT4 has a requisite role in IFNα/β signaling (16).

IL-18 - Biologically active IL-18 is an 18.3 kDa polypeptide, initially termed interferon-γ-inducing factor (IGIF) (3,17-19). The murine and human IL-18 genes were cloned in 1995 and 1996, respectively (19,20). IL-18 is structurally related to the IL-1 family of proteins, but has no significant sequence homology to any known protein (19-21). Like IL-1β, IL-18 is synthesized as a biologically inactive precursor without a typical leader sequence and is proteolytically activated by caspase-1 before release from cells (21-23). Caspases are intracellular cysteine proteases that cleave their substrates at Asp - X bonds (24). Thus, the production of both IL-1 and IL-18 is regulated by caspase-1 (25). However, the precursor of IL-18 is present in cells both constitutively and in an inducible fashion thus distinguishing it from IL-1β, which is only synthesized in an inducible form. In addition, a novel inhibitor of IL-18 functions, IL-18 binding protein, has recently been described (26).

The IL-18R, like the IL-1 receptors, belongs to the Toll receptor family, and is exclusively expressed on Th1 cells and not on Th2 cells, similar to IL-12Rβ2 (27-29). This unique receptor distribution explains why IL-18 specifically propagates Th1 functions in T cells. The IL-18R is composed of two chains: the IL-1 receptor-related protein (IL-1Rrp) and the Accessory protein-like subunit (AcPL) (28,30). The IL-1Rrp is the low affinity receptor (Kd=~45 nM) and the IL-1Rrp and the AcPL complex constitute the high affinity receptor (Kd=100 pM). IL-18 binds directly to the IL-18Rrp, but not to AcPL; hence proposal of the latter as the signaling chain of the IL-18R (30). The intracellular signaling events resulting from IL-18R occupancy are similar to those observed upon IL-1R activation (25). The first event involves recruitment of the adaptor molecule, MyD88 and subsequently the IL-1 receptor-associated kinase (IRAK) (31,32). Activated IRAK was found to form a complex with tumor necrosis factor receptor-associated factor-6 (TRAF-6) (32). TRAF-6 activates NF-kB-inducing kinase (NIK), which in turn activates the IkB (inhibitor protein of NF-kB) kinases- α and β (IKKα and β) (27). IKK activation phosphorylates IkB, which normally sequesters the transcription factor, NF-kB, in the cytosol; phosphorylated IkB is then ubiquinated and degraded by the proteasomal complex in the cytosol. The released NF-kB translocates to the nucleus to initiate the transcription of many genes (33). Interaction of IL-18 and its receptors also activate p56Lck and mitogen activated protein kinase (MAPK) in murine Th1 cell clones (34). It is not clear whether p56lck directly associates with the activated IL-18R. In addition, it is reported that stimulation of IL-18R also leads to activation of the nuclear transcription factor, AP-1, and thereby genes regulated by AP-1 (35). Though some of the IL-18 signalling pathways are shared with IL-1, the existence of other non-overlapping pathways together with the expressions of IL-1R and IL-18R on distinct cell types may explain the differences in IL-1 and IL-18 actions *in vivo*.

The production of biologically active IL-12 and IL-18 are tightly regulated. IL-12 is regulated by the induction of p40 and its pairing with p35 whereas IL-18 is regulated by caspase(s) activation. Both IL-12 and IL-18 are produced by a variety of cell types such as macrophages, dendritic cells, keratinocytes, etc. (for reviews see (2,4,5,25)).

BIOLOGICAL FUNCTIONS OF IL-12 AND IL-18

Alone or in synergy with IL-12, IL-18 induces IFNγ production from T and NK cells. However, IL-18 has a more potent IFN γ–inducing activity than IL-12 on Th1 cells (4). The synergy arises from the ability of each cytokine to upregulate receptors for the other cytokine. IL-12 induces IL-18 receptors on certain T cell sub-populations (36); and IL-18 can upregulate the IL-12β2 sub-unit of the IL-12 receptor (29,36). These two cytokines utilize different receptor-mediated signaling pathways and influence distinct promoter regions on the IFN γ promoter; for example, IL-12 and IL-18 activate distinct AP-1 sites on the IFN γ promoter (35). On the other hand, certain cell sub-populations express IL-18 receptors constitutively and therefore do not require IL-12 for IFN γ production (4,37). Further, the density of IL-18 receptor expression on various cell populations does not correlate with their ability to produce IFN γ (38). This discrepancy could be due to differential expression patterns of the high and low affinity IL-18 receptors on various cell populations.

IL-12 promotes the functional maturation of Th1 cells from naïve T cells and IL-4 serves a similar role for Th2 cells. IL-12 is also an important co-stimulus for the activation of Th1 cells and can downregulate the differentiation of Th2 cells (5). IL-12 knockout mice have reduced, but not absent, Th1 responses such as suppressed T cell IFNγ responses to antigens and DTH reactions (39). The production of IL-12 is enhanced in the presence of IFNγ, thus establishing a positive feedback loop since IL-12 further enhances IFNγ production from T cells. However, IL-12 can also induce the production of IL-10, which can suppress IL-12 production and hence serve as a negative feedback. Hence, IL-12 can have dual regulatory roles in immune responses (5). IL-12 also activates cytotoxic T cells and NK cells and induces IFNγ production from these cells. In addition, it also induces the production of TNFα, IL-2, GM-CSF and IL-8 and to suppress TGF-β production from various cells (5).

IL-18 is also important for the functional development of Th1 and NK cells. IL-18 knockout mice have defects in Th1 and NK cell responses (37). Recent studies show that IL-18 is a potent inducer of (i) TNF, Fas-ligand, and IL-2 from T and NK cells (40,41); (ii) Fas-ligand in a myelomonocytic cell line (42), (iii) GM-CSF from osteoblasts (43,44), and iv) IL-1β, IL-8, and MIP-1α from peripheral blood mononuclear cells (37). In addition to inducing proinflammatory cytokines, IL-18 is a potent inducer of COX2, inducible nitric oxide synthase, and various metalloproteinases from chondrocytes (45). It can upregulate CD40 on macrophages and ICAM-1 (CD-54) on a myelomonocytic cell line (46). Thus, IL-18 is fast emerging as a critical proinflammatory cytokine. *In vitro*, IL-18 synergizes with IL-12 or IL-2 to induce proliferation of either NK1.1-CD3- or NK1.1+CD3- cells, respectively. IL-12 only synergizes with IL-18 to induce proliferation in NK1.1-CD3- cells but not NK1.1+CD3- cells (38,46,47).

Evidence is mounting that IL-12 and IL-18 also have distinct biological actions. The best *in vivo* evidence for the distinct functions comes from IL-12 and IL-18 knockout mice and the use of neutralizing antibodies to these two cytokines. IL-12 and IL-18 knockout mice display partial defects in Th1 and NK cell functions (13,37). The NK cell defects are more profound in IL-18 knockout than in IL-12 knockout mice. However, the T cell and NK cell defects are even more pronounced in the IL-12/IL-18 double knockouts (37). IL-18 cannot induce differentiation of Th1 cells in spleens from IL-12 knockout mice, but can induce IFNγ production by NK cells *in vitro* and increased NK activity *in vivo* (37). These observations suggest that IL-12 is required for Th1 cell differentiation but not for NK cell functions. These studies corroborate findings that IL-12 induces IL-18R on Th1 cells but NK cells

express IL-18R constitutively (4,37). Collectively, it appears that both IL-12 and IL-18 can influence Th1 cell development to some extent and are essential for optimum development of Th1 and NK cell functions (37). However, the precise roles of IL-12 and IL-18 in these processes remain to be elucidated.

Neutralizing IL-12 and IL-18 antibodies demonstrate further distinct *in vivo* effects of these cytokines. Anti-IL-12 antibodies were only partially effective at preventing liver damage in *P. acnes* primed, LPS challenged mice and in down-regulating TNFα and Fas-ligand expression in liver cells (48). By contrast, anti-IL-18 antibodies completely prevented liver damage, late phase of TNFα induction and Fas-ligand expression (48). These observations suggest that *in vivo*, IL-18 also regulates both TNFα and Fas-ligand production. Both TNFα and Fas-ligand play a critical role during inflammatory responses and are potent mediators of cytotoxicity. *In vitro* studies showed that IL-18 induced Fas-ligand expression in Th1 cell clones (41). Further, in a myelomonocytic cell line which does not express IL-12R, IL-18 induced Fas-ligand and apoptosis in an IFNγ-independent manner (42). In addition, IL-18 also induces perforin-dependent cytotoxicity by hepatic NK cells (49). Further, IL-18 up-regulates expression of ICAM-1 (CD54), an adhesion molecule critical in the recruitment of lymphoid cells at the site of inflammation (46). Up-regulation of Fas-ligand and ICAM-1 and perforin-dependent cytotoxic effects of IL-18 are independent of IFNγ and IL-12.

IL-12 knockout mice develop normal Th1-mediated responses to viral infections (50-52). Anti-IL-12 antibodies do not influence development of NK cell activity, IFNγ production, and Th1-dependent responses to viral infections (50). These studies suggest that IL-12 may not be essential for the development of effective anti-viral cellular responses. Similar studies with IL-18 knockout mice or anti-IL-18 antibodies have not yet been done. During viral infections, IL-18 and IFNα/β (but not IL-12) produced by infected macrophages synergize to induce IFNγ (53). This synergy is a result of IFNα/βR dependent activation of STAT-4, which is involved in IL-12 signalling, and up-regulates IFNγ transcription (14,15). These observations suggest that IL-18 can synergize with cytokines other than IL-12 to influence IFNγ production and thereby effect Th1/NK cell development. Similarly, it is possible that IL-12 might synergize with other cytokines, for example IL-1, which shares signaling pathways with IL-18, to induce IFNγ production and modulate Th1 responses (4). Hence, under physiological conditions, the requirement for IL-18 and/or IL-12 to serve as a co-stimuli for IFNγ production and functional development of Th1/NK cells is determined by the nature of the antigenic stimulus and cytokine milieu.

Both IL-12 and IL-18 are essential for effective IFNγ production and efficient clearing of some bacterial infections (54,55). IL-12-deficient or IL-12R-deficient patients are defective in clearing intracellular bacteria during the early phases of the infections (50-52). IL-12 knockout mice and IL-18 knockout mice are both defective in LPS-induced IFNγ production (13). Similarly, caspase-1 knockout mice which fail to produce bioactive IL-18, but make normal levels of IL-12, are defective in LPS-induced IFNγ production and LPS-associated lethality (22). Treatment with either anti-IL-12 or anti-IL-18 antibodies prevented LPS-associated lethality in mice (56,57). These observations suggest that in response to LPS, and perhaps other bacterial antigens, both IL-12 and IL-18 are required for induction of optimum Th1/NK cell functions and production of IFNγ, a critical player in the direct and indirect consequences of bacterial infections (37).

IL-12 AND IL-18 IN IMMUNOLOGICAL DISEASES

Crohn's disease (CD)- Increased expression of IL-12 and interferon-γ has been observed in the intestinal mucosa of patients with Crohn's disease (58). A rat anti-

murine IL-12 monoclonal antibody suppressed TNBS-induced colitis (a CD model) in mice even after onset of bowel inflammation (59). Anti-IL-12 antibodies also suppressed disease according to clinical and histological scoring in other mouse models of colitis (60). IL-18 is produced by gut epithelial cells and may also play a role in inflammatory bowel diseases such as Crohn's disease. Efficacy data using anti-IL-18 antibodies in animal models of inflammatory bowel diseases are not yet available.

Multiple sclerosis (MS)- The mRNA for IL-12p40 (the inducible subunit of IL-12) was found in lesions of MS patients and chronic progressive MS patients have elevated circulating levels of IL-12 (61-63). Neutralizing anti-IL-12 antibodies can suppress experimental autoimmune encephalomyelitis (EAE) in mice that received encephalitogenic T cells by adoptive transfer or in mice with actively induced EAE (39,64). A rat model of EAE was shown to be suppressed with an anti-IL-18R antibody, suggesting that IL-18 may also play a role in MS (65).

Insulin-dependent diabetes mellitus (IDDM)- The pathology of IDDM in mice is dependent on Th1 cells because these cells can adoptively transfer this disease as evidenced by the appearance of insulinitis and hyperglycemia in naïve recipient mice. The chronic administration of IL-12 enhances insulinitis and diabetes mellitus in NOD mice. IL-12 antagonists can similarly suppress IDDM in NOD mice (66). Increased IL-18 and IFN γ expression have been found near islet cells in NOD mice with IDDM and the Idd2 locus for IDDM is closely linked to the IL-18 gene (67).

Rheumatoid arthritis- The inducible IL-12p40 mRNA has been detected in synovia from rheumatoid arthritis (RA) patients (68). Enhanced levels of IL-12 protein were observed in synovial tissues and fluids of RA patients. In addition, a predominantly Th1 profile of cytokine expression was observed using sensitive techniques to measure cytokine mRNA expression in synovial T cells from RA patients (69). IL-12 administration enhances severity of arthritis in the collagen-induced arthritis (CIA) model in mice and IL-12 knockout mice are resistant to collagen-induced arthritis (CIA) (70). Anti-IL-12 antibodies are efficacious in suppressing CIA in mice when given early in the disease (71). IL-18 expression has also been observed in synovia of RA patients (72).

Septic shock- As evidenced in animal studies, IFN-γ is a late mediator of septic shock, appearing after TNF and IL-1 (73). As mentioned above, IL-12 and IL-18 are both necessary for the production of IFN-γ in endotoxemic mice. IL-12 antagonists such as anti-IL-12 antibodies and p40 homodimers prevented lethality in endotoxic shock (5,57). Anti-IL-18 antibodies decreased lethality in animal models of hepatic failure and endotoxic shock, as noted earlier (48,56).

Others- IL-12 has been implicated in the pathogenesis of experimental models of uveitis and contact hypersensitivity (8,9). Increased levels of IL-18 have been found in the sera of patients with acute lymphocytic and chronic myelocytic leukemias and IL-18 could potentially play a role in the pathogenesis of these leukemias (10).

THERAPEUTIC STRATEGIES

Inhibition of Function - The successes in RA of neutralizing TNF antibodies and soluble receptors have strengthened the notion that neutralization of a single proinflammatory cytokine can be beneficial despite the complexity of clinical disease. Neutralization of IL-12 and/or IL-18 functions by monoclonal antibodies, respective receptor-Ig fusion molecules, or natural antagonists [e.g. p40 homodimers for IL-12 and IL-18 binding protein for IL-18], therefore, offer novel therapeutic approaches for the treatment of various acute and chronic diseases (5,26). These approaches have the advantage of high specificity, and speed of development. Further, human anti-human IL-12 and/or IL-18 antibodies and

antagonists could be used for chronic therapy because of prolonged half-lives and/or lack of immunogenicity.

Functions of IL-12 and IL-18 can also be inhibited by blocking receptor-mediated signaling events. Several key molecules in the signal transduction pathways of IL-12 and IL-18 have been identified. Small molecular weight inhibitors of IRAK, NIK and NF-kB activation should block IL-18 functions, while inhibitors of STAT-4 should block IL-12 functions.

Inhibition of Production - Phosphodiestrase 4 (PDE4) inhibitors and β2-adrenergic agents inhibit the production of the p40 subunit of IL-12 by enhancing cAMP levels and protein kinase A (PKA) activity. Prostaglandin E2 (PGE2) also inhibits IL-12 production by the same pathway. However, the exact pathway of cAMP-mediated inhibition of IL-12 production is not known. Production of biologically active IL-18, at least in response to LPS, can be effectively blocked by caspase-1 inhibitors *in vitro* (22,74).

Conclusion -The molecular mechanisms of production and signalling for these two important proinflammatory cytokines have not yet been completely elucidated. The discovery of signal transduction pathways and regulatory mechanisms unique to the individual cytokines offers opportunities for the discovery of small molecule inhibitors to modulate the functions of these cytokines.

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Chapter 23. Genomics, Target Selection, Validation, and Assay Considerations in the Development of Antibacterial Screens

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Introduction - The increasing incidence of antibiotic resistant bacteria has intensified the quest to find targets for new antibiotics. The bacterial genomics initiative is rendering an unprecedented choice of target candidates for anti-bacterial development (1,2). Since the release of the complete genome sequence *of Haemophilus influenzae* in 1995, 18 fully sequenced genomes were published and another 45 genomes are currently being sequenced (1). Comparative genomics provides a basis for creating lists of potential antibiotic targets that are either unique to a single bacterial species, common to a subset of species, or found in a broad spectrum of pathogenic bacteria. However, such lists generated via comparative sequence analysis must be refined to reveal those proteins that are essential to the viability of an organism. These constitute a group of targets of significant commercial interest and are the subject of this review. Proteins selected by an informatics approach and identified as potential targets need to be screened in a biological assay in order to be validated as essential for growth or viability (3). These include proteins of known function and those of unknown function. Configuring assays for proteins of unknown function remains a significant challenge in the search for novel antibiotics and there remain plenty of opportunities for creativity in this endeavor (4). Thus, the current paradigm in the quest for novel antibiotics begins with the annotation and categorization of genomic sequences and leads through the identification, validation and functional evaluation of protein targets that meet specific criteria for assay development. This review focuses on the processes required to transform a DNA sequence into a tool for the discovery of novel chemical leads.

GENERATION AND ANNOTATION OF PATHOGEN SEQUENCE DATABASES

Shotgun Sequencing, Bioinformatics and Genome Annotation - The genome sequences of a substantial number of human pathogens have been successfully obtained through many large-scale whole genome random sequencing or "shotgun" projects (5, 6). It is not necessary to have a complete genome sequence in order to uncover prospective targets. If a 2.5 Mb genome is randomly sequenced to a six-fold excess, the random sequences can be assembled into approximately 50 segments of DNA called "contigs" and to form an ordered map of the bacterial chromosome. After editing for frameshift errors, the complete sequence of most genes is present. On average, one gene is found every 1000 base pairs and the organization of most genes is apparent as well as synteny to other pathogens.

Identifying the open reading frames (ORFs) present in DNA sequences, predicting which ORFs are coding and annotating the function of the encoded proteins in a bacterial genome has become highly automated. Various analysis programs are routinely used to find ORFs and to measure their identity to sequences in other databases throughout the process of finishing and annotating a

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genome (7, 8). In the preliminary analysis of a sequence, basic sequence alignment search tools such as BLAST or FASTA are used to identify high-level sequence similarities between a query sequence and all of the sequences in one or many databases (9,10). BLAST or FASTA identities or "hits" are listed as probabilities, *E* values, which should measure the expected number of sequences in the database that would achieve a given score by chance (8). These values are subject to the quality of the query sequence and the compositional biases of the database(s) being searched. The sensitivity of the search method can be increased by using PSI-BLAST (Position-Specific Iterative BLAST, ref. 11) and the significance of motifs or patterns found in small regions of a protein can be further searched using PHI-BLAST (Pattern-Hit Initiated BLAST: ref. 12). Poorly characterized and newly sequenced genomes can benefit from comparisons to well-annotated genomes such as *E. coli*, which has been subjected to extensive biochemical and genetic analysis (13,14). Bacterial proteins in different phylogenetic lineages have been compared to uncover consistent patterns of sequence similarities allowing the delineation of clusters of orthologous groups or COGs (15). There are web-based tools for performing database sequence searches for identity (8). Such analyses can facilitate the automated functional annotation of genomes. However, it can be misleading to stamp functional annotations on proteins solely on the basis of sequence relationships that are assigned by computational methods (16). Therefore, improvements in the detection and prediction of functional relationships have been proposed (17).

SELECTING ANTIBACTERIAL TARGETS

Scanning Databases for Sequence Relationships among Targets - In order to find targets for new antibiotics, one must find proteins that are common among pathogenic bacteria. Comparative genomics helps categorize related sequences across many different bacterial species. The computational analysis and screening of databases selectively generates lists of proteins that are unique to a specific pathogen or common to a set of pathogens (3, 18). The criteria that are established for a given search will influence the parameters, e.g. "BLAST scores", that must be met if a protein is to be included on a list of prospective antibacterial targets. Interpreting the significance of the data obtained also influences target selection. Refining a search by scanning for specific protein segments or domains helps reduce noise and uncovers information that may be relevant to function. Databases such as PROSITE, PRINTS and BLOCKS contain motif descriptors that specify certain structural or functional features of proteins (17,19). Databases are also being compared and annotated for structural features of proteins such as fold usage (20, 21). Information about protein structure can aid in the identification of functional homologies between proteins (22). The "Sequences Annotated by Structure" website contains a suite of programs that are designed to facilitate the use of structural information in sequence analysis (13,24).

Designing Search Criteria - The experimental design of a query, the databases chosen and the interpretation of the data obtained from a search are all subject to the investigator who poses the questions (3,8,18). If the objective is to find broad-spectrum antibiotics, then a preliminary BLASTP or FASTA search would probably scan the databases of all known Gram-positive and Gram-negative pathogenic bacterial species or a subset of such species. It is possible to address prospective problems that may be related to antibiotic toxicity in man when building a target list. For example, it may be important to eliminate any bacterial sequences that show

significant similarity to human sequences. Because the human genome sequence will not be completed for another few years, one can use, as a surrogate, *C. elegans*, *S. cerevisiae* or the non-redundant databases of human, mouse and rat sequences found at the National Center for Biotechnology Information website to remove sequences with high identities to proteins found in eukaryotic organisms (25). Such a strategy may reduce the risk of developing an antibiotic that might cross-react with a related human protein. Many searches include criteria for the removal of any proteins that bear significant homology to human sequences, although the targets of the majority of marketed antibiotics show some conservation with mammalian proteins.

Broad Spectrum Targets - In order to identify new broad spectrum antibacterial targets, a concordance analysis was used to search for conserved proteins that are common to a set of specified bacterial genomes (18). *E. coli* proteins were matched by FASTA analysis against *B. subtilis, H. influenzae*, *H. pylori* and *M. tuberculosis* using an inclusion criterion cut-off with an E-value $\leq 1.0 \times 10^{-24}$. In addition, any proteins that matched sequences found in *S. cerevisiae* were discarded if homologies produced an *E* value $<1.0 \times 10^{-6}$. By this approach, of the 4289 ORFs in *E. coli*, 1944 proteins matched at least one of the other organisms. By requiring that proteins be present in all four bacterial organisms, 1679 proteins were removed from the set of 1944, and an additional 176 proteins showing homology *to S. cerevisiae* were removed by the exclusionary criterion. Thus, 89 proteins were found to be in concordance across all species. Twenty-three of those proteins have no function assigned to them. Essential proteins of unknown function represent a new class of targets for antibiotic development.

Protein Targets Common to Taxonomic Groups - By scanning the genomes of only Gram-positive bacterial pathogens, it is possible to uncover a set of proteins common to these medically relevant organisms. This list is further refined to remove proteins found in higher organisms, such as man, using the criteria noted above. A second cut through this select set of sequences can exclude proteins with significant homology to Gram-negative bacteria by subtracting sequences with high identities to *E. coli* (13). Each cut serves to increase the likelihood of compiling a list representing sequences specific to "Gram-positive" pathogens.

Species-Specific Target Identification - Specialized criteria are used to identify targets that are unique to a single bacterial species. For example, *H. pylori*, the causative agent of ulcers, is a non-life-threatening infection, and an opportunity exists to develop a user-friendly antibiotic that will not interfere with the growth of microflora inhabiting the gut. The *H. pylori* genome is examined for proteins that share common function in other bacteria, while simultaneously imposing a cut-off criterion to identify only those proteins with a sequence identity so low as to provide the potential for developing an *H. pylori* selective antibiotic. The release of a second genome sequence for *H. pylori* (26) provides a unique opportunity to perform the first complete genome sequence comparison of two unrelated isolates of a bacterial species. Remarkably, the two strains are quite similar with most of the differences clustered in a single hypervariable region termed the plasticity zone. Although strain-specific genetic diversity has been proposed to explain differences in susceptibility to infection by the organism, minimal genetic diversity is observed overall between the two virulent strains. This information will aid the identification of conserved protein candidates for the development of diagnostics, vaccines and antibiotics.

Caveats to Database Searching - Common pitfalls can occur in any search. List output may be skewed if quality control standards are not consistent for all of the databases being searched. If one is searching for "Gram-positive" targets and the database of a single Gram-positive organism is poorly represented, it is possible that some targets will be overlooked. This may happen if a computer query is designed to look for similarities among genomes on an all-or-none basis. If there is poor sequence coverage or incomplete representation of a single genome, the list of ORFs that are found to be in common among all of the other Gram-positive organisms will be reduced by the set of common genes that remain unaccounted in the one incomplete data set. Pre-setting a parameter in the query that requires proteins to be common to only 4 out of 5 genomes will reduce the negative effect of single genome of poor quality. The selection of potential targets can also be masked if a probability score is preset to a cut-off value that is too stringent to include all of the true orthologs that are present in every genome under consideration. Therefore, it may be necessary to conduct a series of searches, each calculating different probabilities designed to provide several sample lists of targets. The composition of members on the target list will change as the qualifications for inclusion are varied. A BLAST search may reveal an identity between proteins in different bacterial species. However, amino acid identities by themselves do not always reflect equivalent function between proteins. Genomic microbiology requires vigilant and consistent assessment of functional relationships that are presumed on the basis of sequence similarity.

Hidden Protein Targets - The functional assignments given to individual proteins can be superimposed onto the genome and we can view the vast schematic diagram comprising all of the metabolic pathways that make up a living organism. Clues that lead to the identification of prospective targets are gleaned from the presence or absence of enzymatic pathways, or gaps that are found where orthologous proteins appear to be missing in otherwise intact biosynthetic pathways. However, when informatics analyses fail to uncover an orthologous protein, it may reflect: 1) elimination of enzymatic activity from the genome, 2) extremely low sequence homology, 3) incomplete database due to sequence gaps, or 4) a protein with a non-identical sequence possesses the same enzymatic activity that is necessary to fill the functional gap in an essential metabolic pathway (non-orthologous gene displacement) (27). For example, *Methanococcus jannaschii* seems to be missing a key metabolic enzyme, thymidylate synthase (TS), which is required for thymidine biosynthesis. However, when a structure-based prediction method known as threading was used to scan all proteins of unknown function in the database, a potential TS gene called ORF MJ0757 was identified, and it is only 7% identical to the amino acid sequence of TS found in *E. coli* (28). It remains to be seen if this represents the TS ortholog in *M. jannaschii.*

Databases can be screened to identify specific protein motifs or families of sequences. FtsZ is an essential gene in *E. coli* and *B. subtilis* and it is one of the most broadly conserved cell division proteins (29,30). FtsZ has been proposed as an excellent target for broad spectrum antibacterial drug development. Up until the release of the *Chlamydia trachomatis* genome, a sequence homologous to FtsZ had been found in every eubacterial species and in archaeal species, as well (31). Should the apparent absence of FtsZ in *Chlamydia* impact the decision regarding whether it constitutes a good broad spectrum target? Another protein in *Chlamydia* may substitute for the function of FtsZ, or an FtsZ-like enzymatic activity may not be required in *Chlamydia.* Theoretically, any protein selected by an informatics

approach may be subject to similar prospects, and biological approaches have their limitations as well. The genome sequence of *Chlamydia* reveals a full complement of enzymes regulating cell wall synthesis, despite the fact that for many years it was a paradox as to how penicillin inhibited *Chlamydial* cell division in the apparent absence of any discernible components of peptidoglycan (31, 32).

VALIDATING POTENTIAL TARGETS

Directed Gene Knockouts - The first step taken in the transition from informatics to the biology of target development is identifying gene products that are essential for the viability or growth of bacteria. Alternatively, essentiality might be viewed as the very first criterion that should be fulfilled when building a list of potential antibiotic targets. Traditional methods for determining whether a gene is essential for the growth of an organism rely on the isolation of conditional lethal mutants or directed-knockout methods. Insertional mutagenesis can produce polar effects in downstream genes. For example, if disruption of a non-essential gene affects transcription of a downstream gene that is essential, then the disrupted gene may be misread as being essential. Refined methods for generating precise deletions in genes have been developed (33).

Genetic Footprinting - High throughput methods use transposon-mediated mutagenesis in conjunction with genetic footprinting to detect essential genes. Footprinting takes advantage of the wealth of genome sequence information. The method, originally developed in yeast, introduces and analyzes mutations on a genome wide scale (34,35). Transposons are introduced into a population of growing cells and transposition is allowed to take place over several generations. From this, a comprehensive library of mutants is generated in which every gene has undergone at least a single mutagenic event and each cell bears only a single mutagenic event. By comparing samples of DNA taken from cell populations before and after mutagenesis, it is possible to determine how disruption of any given gene affects cell growth or viability relative to its environment. To assess whether a particular gene was disrupted and whether that disruption plays a role in cell growth, it is necessary to design a set of primers, one that is specific for the gene of interest and another that is specific for the transposon. In the simplest case, if a PCR product of a gene can be detected after several generations of cell growth, it means that the cell could tolerate disruption of the gene by the transposon and still grow. Thus, the gene is marked as non-essential. When it is not possible to find PCR products representing transposition events in a particular gene, this is taken as evidence that the gene is essential to the growth or viability of the cell.

Genetic footprinting has recently been applied, in modified form, to bacteria by using a method termed GAMBIT (genomic analysis and mapping by *in vitro* mutagenesis)(36). In yeast, transposition takes place over several generations in growing cell populations. By the GAMBIT method, the *Himar1* transposon was used in conjunction with purified *Himar1* transposase *in vitro* to mutagenize bacterial chromosomal regions or PCR products of approximately 10 kilobases in size. Both *H. influenzae and S. pneumoniae* were transformed with repaired transposition products, and recombinant bacteria carrying the genetically modified chromosomal segments were subsequently analyzed by genetic footprinting. Based on their extremely small sampling (a total of 13 genes in two species), the authors predict that approximately one third of all conserved hypothetical genes may be essential for growth (36). Currently, the GAMBIT technology works well in naturally competent

bacteria and can be used to scan small portions of the genome for genes that are likely to be essential for cell viability or growth. Regardless of whether a gene disruption method is based on directed insertions or transposon-based disruption, only a limited number of bacterial systems are sufficiently well characterized to provide the genetic tools necessary for target validation. An alternative to gene disruption is the application of antisense peptide nucleic acid (PNA), which has been shown to inhibit gene function in bacteria and can be used for target validation (37). The antisense PNA method may be widely applicable to many different bacterial species as a means for validating gene targets.

Using Informatics to Enhance the Identification of Essential Unknowns – There are advantages to enriching for novel and conserved targets using informatics. When concordance analysis tools are used to match conserved proteins in *E. coli* or *B. subtilis* with proteins present in other pathogenic bacterial species, approximately 20% of the proteins of unknown function selected by the process are found to be essential (18). Arigoni *et al*, tested the hypothesis that conserved sequences should be associated with functions that are essential for growth (3). They used BLAST analysis to compare *E. coli* proteins of unknown function to the genome of the distantly related intracellular parasite *Mycoplasma genitalium* in order to define a common set of conserved sequences which might represent targets for broad spectrum antibiotic development. Of 40 *E. coli* proteins selected by informatics, 14 sequences with identities to ABC transporters were eliminated. In the end, 26 common highly conserved genes of unknown function, found in a wide range of bacteria, were subjected to in-frame deletions in *E. coli* with the result that only 6 turned out to be essential. Five of the six genes were also essential in *B. subtilis*. This represents a smaller percentage of essential hypothetical proteins than that predicted for *S. pneumoniae* and *H. influenzae* using the GAMBIT technology, as described above (36). The low percentage of conserved essential proteins of unknown function found in *E. coli* was unexpected and might be explained by the presence of additional paralogous proteins, that perform similar functions in the larger genome of *E. coli*. Thus, when screening for essential proteins, the results obtained will be subject to the parameters of the biological system(s) used for study and may not necessarily reflect the growth requirements of every individual organism. Furthermore, labeling a protein as essential for growth based upon cell viability in a petri dish will not necessarily reflect the growth requirements of the organism during growth in its human host. Additional information about the role of genes in cell growth may be gleaned from studies using animal models of infection.

DEVISING ASSAYS FOR SCREENING

Generally, the targets of antibacterial screens fall into two different categories, proteins of known function and those of unknown function, which make up 40 to 60% of the sequences in bacterial organisms. By definition, orthologous proteins retain the same function in different bacterial species. For proteins of known function, the development of a screen can be initiated by searching the literature for established assays and determining how to address questions that are related to conversion of the assay to fit the parameters of the enzyme at hand. Therefore, this section is devoted to the consideration of some screening methods that might be used on genes encoding proteins whose function is unknown.

Phage Display - Cell-free biochemical and biophysical approaches that rely on the direct interaction of ligands with targets work best on soluble or accessible proteins.

Such approaches allow a preliminary assessment of ligand binding without requiring the development of an enzymatic assay or knowledge of target function. Phage display of peptides has been widely studied and the identification of peptide ligands that bind to a variety of proteins has been extensively documented (38). A peptide ligand can provide a stepping stone for studies where small drug-like compounds are searched for their ability to displace the peptide from the target. Affinity selection used in conjunction with mass spectrometry is a direct physical approach and a rapid means of identifying ligands in diverse combinatorial libraries comprised of peptides, or small drugs (39-41). For proteins not amenable to soluble expression, purification and analysis in cell-free systems, the cell-free identification of peptide ligands for proteins of interest has been adapted to an *in vivo* whole cell screening system called selectively infective phage (SIP)(42). The g3p protein of fd phage is split, rendering the phage non-infectious, and a protein of interest is genetically fused to the carboxy-terminus while peptide ligands are fused to the amino-terminal domain (adaptor domain). The adaptor can be modified for fusion with a library of peptidic ligands, or alternatively, non-peptidic ligands can be chemically coupled to the amino-terminal domain (43). Infection, and thus phage propagation, is dependent upon interaction of the ligand binding protein with the ligand, which effectively returns the missing amino-terminal domain to the phage. This method has also been used to select multiple protein-protein interactions from combinatorial libraries (44).

Bacterial Two-Hybrid System - The yeast-two hybrid system, which is linked to transcriptional activation, has been widely used to detect protein-protein interactions as well as protein-peptide interactions *in vivo* (45,46). Unfortunately, some membrane proteins do not work well in this system. In an extension of the yeast two-hybrid method, a bacterial two-hybrid system has been designed to detect protein-protein interactions in a simple genetic screen (47). Complementary fragments of the catalytic domain of adenylate cyclase, CyaA, from *Bordella pertussis* were fused to a series of interacting proteins, each of which functionally restored adenylate cyclase activity. When this system was expressed in an adenylate cyclase deficient strain of *E. coli*, the resulting synthesis of cAMP enabled the fermentation of lactose or maltose, which was used to monitor the growth of colonies. It has been proposed that the bacterial two-hybrid system could be used to screen interactions between a library of DNA-encoded peptides and a "bait" protein (47). A peptide inhibitor of *E. coli* Prolyl-tRNA synthase was found to drastically inhibit bacterial growth *in vitro* using a technology called VITA (Validation *in vivo* of Targets for Anti-infectives) (48).

Screening Small Molecule-Protein Interactions in Cells – Whole cell screening of compound libraries provides a wealth of information about pharmacological effects that cannot be predicted *in vitro*. Nanodroplets carrying beads linked to compounds synthesized by split pool synthesis mixed with finite numbers of cells have been proposed as a means for detecting protein-ligand interactions on a large scale (49, 50). Using a miniaturized array assay format (6,500 assays in a 10 cm petri dish), the biological effects of a small molecule, in this instance, rapamycin, were detected by measuring the inhibition of yeast cell growth after the compound was released from the beads by photo-cleavable linkers. Nanodroplets have also been used to select inhibitors of protein-protein interactions in yeast. This is accomplished using yeast carrying vectors for the reverse two-hybrid system (51). The yeast reverse two-hybrid system uses a toxic reporter gene as negative selection for detecting protein-protein interactions (52). A similar read-out has been proposed for the bacterial two-hybrid system, where disruption of a given interaction would facilitate

the screening of chemical compounds (47). Further development of these methods has potential application in the functional screening of individual cells against ordered libraries of cell-permeable small molecule ligands.

Genome-Wide Transcription Profiling - Microarray technology enables data collection on a grand scale and informational databases are being built to track the impact of genetic, chemical and physical changes across the entire genome (53). Bacterial mRNA has been profiled in high density microarrays (54). Thus, we may soon view the transcriptional activity *in toto* of a pathogen in response to drugs. This powerful technology was used to profile the differential expression of nearly 6,000 genes in *S. cerevisiae* (55). Wild-type yeast treated with the drugs FK506 and cyclosporin A were compared to yeast strains carrying deletions in the gene calcineurin, which encodes the known target of the drugs. The "signature patterns" of gene expression were compared for the different strains revealing target-specific pathways that were altered by the drug in wild-type cells and that were predicted by genetic deletions in calcineurin mutants. Drug treatment of various mutant strains also provided information about drug effects that were mediated through unintended targets. Every yeast gene is being disrupted by the *Saccharomyces* Deletion Consortium (56). This will lead to the collection of expression profiles for all of the deletion strains. The expression profiles determined for drug-treated cells can eventually be compared with expression profiles of genetic mutants, which will provide a convergence of information about the specificity of drug inhibition and potential toxicity in cells. Microarrays of bacterial genes will enable similar studies and may help elucidate novel pathways for antibiotic treatment.

Screening Entire Metabolic Pathways - To screen for agents that will target multiple proteins in a specific metabolic pathway, a general readout could be formulated around a single enzyme in the pathway. Microarray results could provide the data to set-up such assays. For example, the secretion pathway of bacteria contains a number of essential proteins and one of the central components, SecA, is induced in the presence of secretion inhibitors. Therefore, agents that inhibit e.g. SecE, SecY or SecG should also induce the expression of SecA. A secA/lacZ fusion reporter construction has been used in *E. coli* in screens of synthetic compound and natural product libraries, and a number of bona fide secretion inhibitors were identified (57).

FUTURE DIRECTIONS

The array of new protein targets, their rapid rate of discovery through genomics and the demand for high throughput screens poses interesting challenges for the design, composition and screening of compound libraries. The range of enzymatic functions found in new targets should be equally matched with compound libraries that contain sufficient molecular diversity to permit the discovery of novel classes of antibiotics. Combinatorial chemistry has revolutionized medicinal chemistry by applying new methodologies in design and high throughput chemical synthesis. New methodologies emerging from the development of combinatorial libraries are intended to work in synergy with genomics to increase the probability of finding leads (58). Information on the enzymatic mechanism or structure of a protein is used to optimize combinatorial synthetic schemes to create focused libraries with an increased likelihood of containing compounds that hit their targets (59). The three-dimensional structures of proteins serve as templates for the computational screening of virtual compound libraries (60). On the other hand, it may be advantageous to screen essential proteins of unknown function against large

numbers of compounds in highly diverse combinatorial libraries, thus increasing the probability of finding a hit. There are additional proteins essential to bacterial pathogenesis beyond those defined using the criterion of growth on a petri dish. Proteins such as those regulating initiation and maintenance of virulence of different species may prove to be excellent targets for the development of antibiotics or vaccines (1,2,61). Strategies that effectively merge the ideal targets with compatible compound libraries for evaluation in robust and sensitive assays should accelerate the process of lead identification. As we approach the new millennium, it seems fitting that all of this could be done faster and more efficiently.

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Chapter 24. The Case for Recombinant Production of Pharmaceutical Proteins in Plants

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Introduction – Will protein pharmaceuticals produced in transgenic plants in the near future become available for medical use? At present, indispensable pharmaceutical proteins such as insulin, interferon and erythropoietin (EPO) are almost exclusively produced in fermentation reactors. These were first developed in the 1970s in conjunction with genetic engineering techniques and they primarily relied on *Escherichia coli* as a production host organism. However, driven first by the limited repertoire of recombinant proteins that can be produced using *E. coli* and then by the need to produce adequate amounts of proteins in an economical fashion, alternative production systems were sought. These alternatives included *Saccharomyces cerevisiae* (baker's yeast) and various other eukaryotic cell culture systems. The horizon of manufacturing strategies was widened further with the development of transgenic animals and plants as host systems; and the efficacy of the first recombinant protein pharmaceuticals obtained from the milk of transgenic mammals is currently being assessed in late stage clinical trials (1). The use of transgenic plants as sophisticated bioreactors for the expression of recombinant proteins has advanced to a stage where the first clinical indications have been evaluated (2,3). A pharmaceutical industry built, at least in part, on an agricultural base, rather than on large-scale cellular fermentation technology, has become a serious prospect.

In this chapter we evaluate the promise of plants to provide clinically relevant proteins. We will not attempt to offer an all encompassing overview of the scientific state of the art, as in recent years several excellent reviews have appeared (4,5). Instead, we will provide specific examples of two classes of plant-produced recombinant proteins. In addition, this chapter will highlight several critical issues which, from a commercial perspective, will dictate when and to what extent plant produced pharmaceutical proteins will become available to clinicians.

PLANT MOLECULAR FARMING

Gene transfer technology is now routinely practiced to manipulate foreign protein expression at the molecular level in a wide variety of plant species, including all major food and feed crops. A recent review indicates that well over 50 transgenic crops are under development (6). Initial commercial efforts harnessed plant genetic engineering technology to enhance the performance of agriculturally important plants by introducing traits such as pest- and herbicide-resistance. As a result, several genetically engineered crops are presently grown on a commercial scale. According to a recent estimate, in 1998, the global area planted with transgenic crops was over 75 million acres and is steadily increasing (7). These successes have created an interest in further exploiting the capacity of plants to produce valuable products. In one alternative branch of applied plant genetics, the plant is in essence a solar-powered bioreactor refined via genetic engineering to express desirable macromolecules, such as pharmaceutical proteins. This "molecular

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farming" approach is largely underpinned by the same enabling genetic engineering technologies as those used to improve agronomic traits. To date, expression of a considerable variety of proteins for pharmaceutical purposes has been demonstrated. These proteins include: blood serum proteins (8,9), blood anti-coagulants (10,11), human lysozomal proteins (12,13), autoantigens (14), therapeutic toxins (15, 16) and small bioactive peptides (17, 18). To illustrate significant recent scientific progress, we consider here the expression of antibodies and vaccines in plants in some detail.

PHARMACEUTICAL PROTEINS EXPRESSED IN PLANTS

Monoclonal antibodies and antibody fragments – Antibodies and antibody fragments have significant pharmaceutical value because they may, for example, be used as cell type-specific delivery vehicles for therapeutic or diagnostic molecules in the treatment of many diseases including cancer (19). Monoclonal antibodies may also be used for passive immunization, for example, to prevent infections in the oral cavity and to prevent sexual transmission of disease (20,21).

The first report describing the production of antibodies in plants demonstrated the stable expression of heavy γ (Hγ) and light κ (Lκ) immunoglobulin chains of a monoclonal IgG1 antibody in two separate transgenic tobacco lines (22). The antibodies, when assembled in a Hγ–Lκ complex, exhibit binding to low molecular weight synthetic phosphate esters and catalyse the hydrolysis of certain carboxylic esters. Remarkably, accumulation and assembly into an antibody functionally equivalent to a mammalian cell derived antibody was achieved in the progeny by sexually crossing the individual tobacco lines expressing the Hγ and Lκ proteins. Antibodies from both plant and mammalian sources showed similar binding to the phosphate esters. These results clearly demonstrated that plants are capable of the assembly of functional multimeric proteins.

A comparable approach to the production of secretory antibodies was undertaken to express the secretory murine antibody Guy's 13 (23). Secretory immunoglobulins are abundantly present in mucosal secretions, such as the gastrointestinal tract, where they form the first line of defense against invading pathogens. Guy's 13 recognizes an epitope on the surface adhesion protein of *Streptococcus mutans* and prevents the development of dental caries (24). Secretory antibodies are more complex than serum antibodies. They comprise two monomeric heavy and light immunoglobulin units dimerized by a joining polypetide (J-chain). Together with a fourth polypeptide, termed the secretory peptide (SC), an oligomeric complex is formed. Production of the SIgA molecules required successive sexual crossing of four transgenic tobacco lines each expressing the heavy chain, the light chain, the J-chain or the SC-peptide. The assembled polypeptide isolated from the progeny recognized the native streptococcal antigen I/II cell surface adhesion molecule. In a subsequent clinical study, Ma and his co-workers showed that the plant produced secretory antibody provided protection against oral streptococcal colonization in humans, while no clinical adverse affects were demonstrated (2). Therefore, these studies are the first indication that plants are capable of the production of antibodies with therapeutic benefits in humans.

Antibodies can be engineered to produce smaller functional polypeptides. Several of these engineered antibodies have also successfully been expressed in plants, including a single domain antibody, single-chain Fv molecules and Fab (25-28). The production of antibodies in plants has recently been reviewed in detail by Ma and Hiatt and by Ma and Hein (29,30).

Vaccines – The concept of vaccine production in transgenic plants was first introduced by Mason et al. (31). The effort was stimulated by the need to produce large quantities of low-cost vaccines. In addition, it was suggested that the production of vaccines in edible plants or plant parts may offer convenient immunization strategies.

A gene encoding a hepatitis B surface antigen B (HBsAg) was expressed in tobacco plants and virus-like particles (VLPs) were isolated from plant leaves. The purified HBsAg particles were shown to be physically and antigenically similar to the HBsAg derived from human serum and to HbsAg from recombinant yeast, the current commercial source of Hepatitis B vaccine (called Recombivax®; Merck, Sharpe and & Dohme), suggesting fidelity of protein folding. In a follow-up study, mice were parenterally immunized with the tobacco derived HBsAG and it was shown that the antibody response mimicked the response obtained with the commercial HBsAg vaccine produced in yeast (32). In addition, lymph node T-cells obtained from immunized mice were shown *in vitro* to proliferate by the tobacco derived HBsAg. Taken together these studies indicate that recombinant viral antigens can be produced in transgenic plants that functionally mimic effective vaccines produced in a yeast system.

Additional viral antigens which have been expressed in transgenic plants for the purpose of vaccine development include peptides derived from Norwalk virus, the cause of epidemic acute gastroenteritis, foot-and-mouth disease virus, human rhinovirus and human immunodeficiency virus (33-36).

Potential recombinant vaccines for bacterial diseases have also been produced in plant systems. Enterotoxigenic *E. coli* causes acute watery diarrhea by colonizing the small intestine and producing one or more heat-labile multimeric enterotoxins (LT). Haq et al. produced a peptide of heat-labile enterotoxin B (LT-B) in tobacco and in potato tubers (37). Mice fed with fresh transgenic potatoes were shown to develop a immune response against LT-B. In subsequent clinical studies, humans, who were fed transgenic potato expressing the LT-B subunit, developed mucosal and systemic immune responses against LT-B (3). This work demonstrates that plant produced antigens are capable of eliciting an immune response in humans. Demonstrating protection against a challenge of Enterotoxigenic *E. coli* will be the next logical step in this work.

Other non-viral pathogenic antigens which have been expressed in plants for the purpose of developing a vaccine include malarial epitopes in tobacco and cholera toxin B in potatoes (38,39).

THE NICHE FOR PLANT PRODUCED BIO-PHARMACEUTICALS: THE COMPETITIVE POTENTIAL

The examples cited in the previous section illustrate that the production of recombinant pharmaceutical proteins in plants is now unequivocally established at a "proof-of-principle" level and extends to a wide range of proteins. However, with a number of robust and versatile expression systems currently present on the clinical protein manufacturing market, it is reasonable to ask, if, from a commercial perspective, there are benefits that plant production systems offer over the conventional systems. In order to address this question, it may help to consider what features an ideal recombinant protein production system should include. Such a system would: (i) allow flexibility in expressable proteins in convenient delivery vehicles; (ii) be inexpensive regardless of the quantities of protein that are required;

and (iii) be safe for the intended human therapeutic uses. The extent to which plant-based systems can meet these requirements relative to systems presently in use or under development will determine their ultimate commercial success.

Flexibility in expressable proteins - The design of the plant expression systems that have so far been developed varies considerably. Some of the peptide antigens referred to above were produced as stably inherited traits by the introduction of a peptide-encoding gene into the plant genome using *Agrobacterium*-mediated transformation. Others were introduced transiently using a plant viral vector such as cowpea mosaic virus (CPMV) or tobacco mosaic virus (TMV) as the carrier of the antigenic determinant (33,38). Further refinements provide precise control over the time of expression of the target gene or the tissue localization (12,40). Expression levels in excess of 1% of total soluble protein are attainable, although they appear to vary considerably depending on the exact expression construct used (15,41). While not all of the plant expression platforms that have been developed are capable or ideal for the production of all types of proteins, no arguments have been put forward for inherent reasons that would preclude success for certain classes of proteins. In this context, perhaps the last major caveat in our understanding concerns plant glycosylation patterns. Like mammalian proteins, many plant proteins are modified following translation. One of these modifications involves the glycosylation of polypeptide. Both plant and mammalian proteins are initially glycosylated in the endoplasmatic reticulum, resulting in the addition of a high-mannose core. Further processing in the Golgi complex and other organelles results in the addition of particular glycan groups, some of which are unique to plants. The final protein glycosylation patterns are important because activity as well as pharmaceutical efficacy of the protein may require glycosylation. At this time, one cannot predict the extent to which activity and efficacy is affected by differential glycosylation patterns resulting from plant expression. In general, one can expected that those plant expression systems that predictably accommodate the production of defined classes of valuable proteins will commercially be the most successful.

Plant systems may be particularly attractive for use in medical applications that do not require that the pharmaceutical protein is delivered in a pure form. In these applications, it is envisaged that the plant tissue confers desirable properties that facilitate delivery of the recombinant protein. Fruits and other edible plant tissues, for example, have been proposed as delivery vehicles for vaccines (31). The economics and logistics for such an application may be ideally suited for developing countries where cold-storage and transportation are lacking. Other plant tissues that may confer desirable delivery characteristics include seeds and vegetative storage organs, such as tubers. Having evolved as a storage organelle for large quantities of protein, the seed is an attractive target organ for the expression of heterologous proteins. Seeds also offer localization in an aseptic environment away from contamination with pathogens, high stability, cost-efficient long-term storage as well as easy transport. One consideration that has remained relatively under-emphasized, is that in order to control the dose of delivered protein, a pharmaceutical distribution network for edible plants will be required. Separation between the pharmaceutical plants and those intended for every-day consumption will require the presence of a well-organized infrastructure. Besides oral delivery benefits, certain plant tissues may also provide a means for topical delivery for burns, wound-healing and other dermatological applications.

Cost of Production - The overall protein production costs will be a key determinant in the applicability of plant bioreactors. Because plants are extremely efficient producers of biomass, essentially needing only water, sunlight and mineral salts from the soil (or fertilizers), the input costs are substantially lower than those

required for any other system. In addition, the existence of an established agricultural infrastructure for growth and harvesting of plant material is beneficial. It is therefore likely that the most significant capital requirements will be downstream purification costs. It has been suggested that typical downstream purification costs for plant material are relatively high because the mixture for downstream processing is complex relative to the mixture obtained from secreted fermentation systems (4). In order for plant applications to proceed to commercial levels, downstream production costs must therefore be offset by the low input costs. In this context, those applications that require little purification, such as edible vaccines, or technologies that offer inherent purification advantages will be particularly attractive from an economic perspective.

One system that has the potential to reduce downstream purification costs is the oleosin expression system developed by van Rooijen and Moloney (42). The protein of interest is produced in seeds as a fusion protein with a plant oleosin and targeted to seed oil storage organelles known as oil bodies. Removal of greater than 90% of the contaminating proteins is accomplished by separating the oil bodies from the aqueous phase using a simple centrifugation step. Enrichment levels are comparable to those accomplished by bacterial and yeast secretion systems. The blood anti-coagulant hirudin was produced and purified from oil bodies in two chromatography steps to near homogeneity, thus demonstrating the commercial applicability of this system (10).

A further cost-advantage that plants offer is that scale-up to virtually any capacity is straightforward, requiring only an increase in the acreage of transgenic plants. In contrast, scaling-up fermentation systems can be cumbersome and typically involves large capital outlay. As a corollary of the scalability of plant systems to produce proteins in very large volumes, plants may be the only practical production system. One example of a high-volume protein with medical applications are biopolymers, which may be employed in applications such as the prevention of post-surgical adhesions, tissue reconstruction and programmed drug delivery. Successful production of elastin in tobacco has been reported (43).

Clinical safety - Safety for human therapeutic use is obviously an important consideration. In this respect, plant systems appear to compare favorably to other systems. The possibility that pharmaceutical products derived from mammalian cell cultures and transgenic animals are contaminated with viruses, prions, mycoplasmas or other hazardous substances raises safety concerns. Monitoring and removal of these substances is required in order to guarantee product safety. As plants share very few pathogens with humans, plants may represent a safer alternative relative to mammalian cell culture or transgenic animals.

COMMERCIAL PRODUCTION OF PLANT PRODUCED BIO-PHARMACEUTICALS: THE CHALLENGE

The above discussion indicates that plants represent a feasible alternative system for the production of a broad range of pharmacologically active proteins from a technical perspective. We have also suggested that by virtue of several unique biological features, plants could be competitive against existing protein expression systems. However, to realize the ambition of a pharmaceutical industry that produces recombinant plant derived products for clinical use, several commercial challenges remain to be resolved.

The commercial milieu - The initial wave of applied agricultural biotechnology research originated at academic institutions and small private biotechnology-

focused enterprises. However, the ultimate commercialization process of recombinant cultivars was by and large orchestrated by multinational agrochemical companies. The molecular farming effort is now largely driven by a new wave of small independent agricultural biotechnology companies. In order to bring pharmaceutical products to the market place, partnerships will need to be formed between existing pharmaceutical and molecular farming companies. Typically, the molecular farming organization will bring technology and technical know-how to the alliance, while the pharmaceutical company will provide clinical expertise, product distribution channels and large-scale manufacturing expertise. The effectiveness of these partnerships between agriculture and the pharmaceutical industry, two historically separate industries, will determine the ultimate success of molecular farming technology. In this context, the extent to which pharmaceutical companies will be open to novel production technologies in view of commitments to existing manufacturing capabilities will likely be an important issue.

Intellectual property rights – The patent system provides an elegant legal mechanism by which a state authority grants to the inventor a unique set of exclusive rights to make, use and sell the invention for a limited time. In exchange, the inventor agrees to publicly disclose his invention. The rationale is that public disclosure stimulates competition and initiatives to "design around" the patent, thus advancing the state of the art which in turn, ultimately benefits the society. The precise extent of the inventors' rights is determined by the patent office. While the owner of a patent has the right to exclude others from practicing the invention, it does not automatically guarantee the freedom to use the invention. To practice the invention commercially, access to patent rights owned by third parties may be required. At present, several broad enabling technologies, such as plant transformation technologies, selection markers and methods of using them and plant promoters and methods of using them, which may be required to practice various molecular farming technologies, are protected by patent rights. The extent to which plant molecular farming organizations (directly or through the formation of partnerships) will be able to negotiate access to all the intellectual property rights they need to practice the desired technology, will be critical to their commercial viability. On the other hand, the rights to the recombinant production methods for several proteins, the US patent rights to the production of insulin, for example, do not extend beyond microbial production methods (44). In a number of cases the production methods rights are still in force when the rights to the protein itself expire. In these cases, plants might be an alternative to organizations wishing to participate in a market for a pharmaceutical protein but lacking acces to microbial production rights.

Regulatory considerations – As novel production hosts, plants will need to be assessed within a regulatory framework that remains to be largely established. Fortunately, the governmental public health agencies administering regulatory policies have accumulated considerable experience from microbial and cell culture systems. In addition, pharmaceuticals derived from non-transgenic plants, such as taxol have been approved and are presently available for therapeutic use (45). Among the regulatory issues that need to be considered are: genetic stability of the source material, the presence of microbial contaminants, product purity and bioequivalence as compared to counterparts produced or purified from other systems (where available). In the United States, the Food and Drug Administration (FDA) has issued "Points to Consider" documents, which may be updated as scientific knowledge accumulates. The "Points to Consider" document that has been drafted for the transgenic animal technologies may serve as a starting point from which plant regulatory considerations can be further developed. Obviously there will be regulatory issues unique to plants. For example, the potential presence of

herbicides and pesticides in production intermediates will require a unique set of guidelines. In this particular case, the long history of use of these molecules will likely help design strategies to monitor removal of these molecules where deemed appropriate. Regulatory considerations unique to plants have been discussed in considerable detail by Miele (46).

Besides safety for human use, environmental impact is an issue, with some considerations unique to plants. Where the quantities of protein required are relatively low, it may be possible to harvest the crop in a contained environment. However, for most envisaged applications, containment will be impractical and other safety strategies must be employed. Among the strategies available are isolation from neighboring crops, the use of self-pollinating plants, the use of male sterile lines and easily recognizable volunteers. Again, the experience obtained from regulating the transgenic crops with improved agronomic properties will likely provide a wealth of information to serve as guidance for the establishment of appropriate regulatory control.

The establishment of the appropriate regulatory framework should be based on sound scientific judgement and responsible and sensible management of the associated health and environmental risks. In all cases, regulatory strategies will have to be developed based on a dialogue between the manufacturers and the appropriate governmental agencies.

Public acceptance – Frequently, the introduction of new technologies leads naturally and understandably to cautionary reactions from the public. In fact, one of the most unpredictable factors determining the ultimate success of the molecular farming industry in general may prove to be the consumer acceptance of molecular farming-based products. While approval by governmental organizations will likely help in gaining public acceptance, credible experts play an important role in communicating and educating the public, so that informed decisions can be made which are based on the real benefits and risks associated with the use of a product. Results from polls on genetic engineering suggest that medicinal applications are generally less controversial (47). There may nevertheless be public reservations depending on the exact product. From the public perceptive, plants may be less controversial as pharmaceutical production vehicles than transgenic animals.

CONCLUSION

There is now little doubt that from a technical viewpoint the production of a wide range of pharmaceutical proteins in plants is feasible. Plant-produced vaccines and antibodies have successfully completed early stage clinical trials, indicating potential human use of these proteins. The extent to which the pharmaceutical industry will come to rely on these products and the extent to which they will become available for human use is now largely dependent on the commercial competitiveness of plant based expression systems against more conventional protein production systems. Plant-based expression systems offer several unique advantages ranging from the ability to facilitate delivery of proteins to potentially more favorable production economics. It appears that these advantages put plant bioreactors in a viable commercial position. However, a number of issues including patent rights, regulatory and public acceptance issues must be resolved before the first plant molecular farming products will appear on the market.

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Chapter 25. New Targets in Cell Cycle Regulation

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Introduction – A better understanding of the mechanistic events that regulate cell cycle progression and cell replication has provided targets for the discovery of novel therapeutic agents for the treatment of diseases that result from uncontrolled cell proliferation (1-3). Enhanced ability to modulate the signaling and regulatory pathways may lead to a greater understanding of cell cycle progression and to valuable advances in clinical care. Cell cycle progression can be viewed as a sequence of events that are regulated by a cascade of protein kinases which are controlled by four known mechanisms: protein-protein interactions (e.g. cyclin-dependent kinases (Cdks) and cyclins), phosphorylation, intracellular sequestration, and proteolytic degradation of the kinase or its regulatory components. Of the four regulatory mechanisms, those involving enzyme catalysis will be the subject of this review because small molecule inhibitors remain the focus of most approaches to therapeutic intervention. Approaches that modulate protein-protein interactions in a therapeutic setting remain less accessible than those that take advantage of small molecule inhibitors of protein kinases, phosphatases, and ubiquitin-ligases. Indeed, two small molecule inhibitors of Cdks, flavopiridol and UCN-01, have progressed into clinical trials and are highlighted below. Since other recent works have reviewed transitions in the early stages of the cell cycle (Cdk2, Cdk4, Cdk6 in G_1 progression, G_1/S transitions, and S phase progression) and small molecule inhibitors (4-6), the emphasis of this review is on molecules that regulate the G2/M transition and M phase progression.

COMPOUNDS IN CLINICAL TRIALS

Two compounds that inhibit cyclin dependent kinases have entered clinical trials. Although little efficacy data have been released, we will report briefly on the characteristics of flavopiridol and UCN-01 (7-hydroxystaurosporine).

Flavopiridol - Flavopiridol is a Cdk inhibitor currently in clinical trials. The compound is a competitive inhibitor with respect to ATP and blocks the activities of Cdc2, Cdk2, and Cdk4 with IC_{50} values in the range of 300 to 400 nM and no apparent selectivity. However, unlike UCN-01, flavopiridol lacks significant activity towards protein kinase C (PKC). The co-crystal structure of Cdk2 and the des-Cl analogue of flavopiridol showed that the aromatic heterocycle occupied the adenine pocket of the ATP binding site (8). In two human breast tumor cell lines (MDA-MB-468 and MCF-7), Cdk2 activity was inhibited after 24-hour treatment with 300nM compound. MCF-7 cells also showed inhibition of Cdk4 activity. Mutant MDA-MB-468 cells (lacking Cdk4-cyclin D) were primarily blocked in G_1 after 24 hours (9). These data suggest that cell cycle arrest may be a primary effect of the inhibition of Cdks by flavopiridol. Much additional biochemical and cellular characterization of the effects of this compound have been previously reviewed (5,6).

In vitro and *in vivo* evaluation of flavopiridol showed that the compound was more active in prostate carcinoma models than in small numbers of colorectal carcinoma, large cell or small cell lung carcinoma, mammary carcinoma, melanoma, ovarian carcinoma, or renal carcinoma cell lines (10). Another study reported tumor-free survivors in HL-60 promyelocytic leukemia and increased growth delay in two lymphoma xenografts (11).

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The initial Phase I trial of flavopiridol utilized a 72 hour continuous infusion schedule every two weeks (12). Dose limiting toxicity of secretory diarrhea was found at 62.5 mg/kg/day x3. In about 70% of the 76 patients in this study who were dosed at 50mg/kg/day x3, a mean plasma concentration of 271nM flavopiridol was measured, consistent with the required amount to inhibit Cdks *in vitro*. One patient with renal cancer showed a partial response and 3 patients with renal cancer, colon cancer, and non-Hodgkin's lymphoma showed minor responses.

Although there are no reports of Phase II trials that examine the combination of flavopiridol with other marketed agents, several *in vitro* studies have been done to examine the combined effects of this and other currently used drugs. When measured by the ability of human A549 non-small cell lung carcinoma cells to form colonies on plastic, flavopiridol showed synergistic effects with seven of eight anti-tumor agents but only when it was administered after the anti-tumor agents (13). This required order of addition was also reported for combined treatment with mitomycin-C in a gastric carcinoma cell line and a breast carcinoma line (14). These studies highlight the necessity of careful dosing schedules in the design of combination clinical trials. Such treatment also induced apoptosis, suggesting cytotoxic mechanisms in addition to cell cycle arrest (15-17).

UCN-01 - UCN-01 is another compound with Cdk inhibitory activity now in clinical trials. It was initially described as an inhibitor of PKC (18) but subsequent investigations revealed that PKC inhibition was not the primary mechanism of its antiproliferative action (19). UCN-01 inhibited Cdc2, Cdk2, and Cdk4 with IC_{50} values of 30nM in enzymatic assays (20). However, in camptothecin-treated HT-29 cells subsequently treated with UCN-01, there was no blockage of Cdc2-mediated histone H1 phosphorylation (21). These data suggest that UCN-01 may directly inhibit Cdk2 but not in the presence of DNA damage. Recent studies suggest that UCN-01 may modulate the G_2-M checkpoint. Based on positive results *in vitro* and *in vivo*, a clinical trial was initiated (22). Early data from 72 hour continuous infusions at 2 doses in humans showed substantial binding of UCN-01 to human alpha-1-acidic glycoprotein and long plasma half-lives (489-915 hours). In humans, the peak plasma concentration of 6.7μM at the highest dose tested in this study was not associated with any reported toxicity. This high circulating level contrasts with studies in dogs, where a maximum tolerated dose resulted in a peak plasma concentration of 0.33μM. Because of the unexpected plasma protein binding and prolonged half-life in humans, the clinical trials have been modified to reduce the administered dose and to extend the time between courses to allow observation of effects that result from drug accumulation. At this point, the clinical trials of this compound appear to be continuing.

REGULATION OF THE G_2/M CELL CYCLE TRANSITION

Extensive genetic and biochemical studies have identified the Cdc2-Cyclin B1 complex as the major regulator of the G_2 to M phase transition (reviewed in 23). Cdc2 protein levels are relatively constant through-out the cell cycle while cyclin B1 protein levels only increase during S and G_2 (24). During S and G_2, the cyclinB1-Cdc2 complex, which accumulates primarily in the cytoplasm, is inactive due to the phosphorylation of two residues (Thr14 and Tyr15) on the Cdc2 catalytic subunit (25, 26). The Myt1 kinase phosphorylates predominately Thr14 and, to a lesser extent, Tyr15 on Cdc2, while the Wee1 kinase phosphorylates Cdc2 exclusively on Tyr15 (27, 28). In mammalian cells, inhibition of Cdc2 activity is believed to play an essential role in preventing mitotic initiation until earlier cell cycle events, such as cell growth, DNA replication, and centrosome duplication have been completed. Several recent reports also demonstrate that Cdc2 Thr14 and Tyr15 phosphorylation plays an essential role in the G_2 DNA damage checkpoint response (see below). At the end of G_2, the Cdc2-Cyclin B1 complex is translocated into the

nucleus where it becomes active owing to Thr14/Tyr15 dephosphorylation by the Cdc25B and Cdc25C dual-specific phosphatases (29, 30). At the end of mitosis, cyclin B1 is rapidly proteolysed by a ubiquitin-dependent process.

Conceptually, inhibition of Cdc2-Cyclin B1 kinase or Cdc25C phosphatase activities should arrest human cells in G_2. However, since there is little evidence supporting a connection between regulation of these particular G2/M cell cycle regulators and cancer, the therapeutic value of targeting Cdc2 or Cdc25C is unclear, i.e. normal cells would be arrested in G_2 just as effectively as cancer cells. However, a therapeutic value of inhibiting kinases that repress mitotic initiation can be envisioned in light of recent progress in understanding the DNA damage checkpoint response pathways.

G_2 DNA DAMAGE CHECKPOINT CONTROL

Most eukaryotic cells delay progression from G_1 to S and G_2 to M following damage of the DNA. Depending on the cell type and damage, these delays (also known as checkpoints) may induce either a transient arrest (31), permitting repair mechanisms time and access to correct the damage before DNA replication or mitosis proceed, or an apoptotic-mediated cell death. In many cases, the G_1 and G_2 checkpoint arrests are mediated by direct inhibition of cyclin-Cdk activity. For example, the p53 gene product mediates G_1 arrest by inducing transcription of the Cdk inhibitor p21 CIP1/WAF1, while the G_2 arrest is dependent, in part, on phosphorylation of residues Thr14 and Tyr15 on Cdc2 (32-35). Since many tumor cells lack p53 function, therapeutic value may be gained by inhibiting components of the G_2 DNA damage checkpoint, i.e. p53 deficient tumor cells may exhibit enhanced sensitivity to DNA damaging agents when also exposed to a Myt1/Wee1 inhibitor (Figure 1) (36). While targeting the catalytic activity of the Myt1/Wee1 kinases is likely the most direct approach towards abrogating the G_2 DNA damage response, other recently reported components of this checkpoint mechanism are worth consideration.

A.

	DNA Damage Response		
Genotype:	G_1 Arrest	G_2 Arrest	Viability
Wild-type	+	+	+
$p53^-$	-	+	+
Cdc2-P-TY^-	+	-	+
$p53^-$, Cdc2-P-TY^-	-	-	-

B.

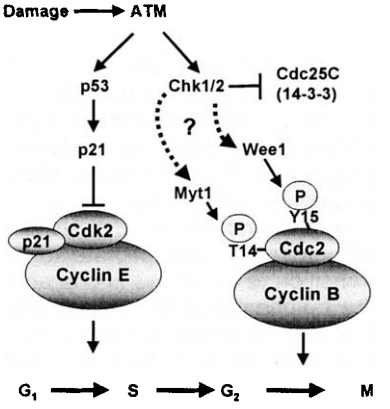

Figure 1. Targets of the G_2 DNA damage checkpoint.

Chk1 - In fission yeast, a genetic pathway consisting of numerous genes including rad3, chk1, and rad24/rad25 has been identified in the G_2 DNA damage checkpoint (reviewed in 37). The rad3-encoded kinase shared significant sequence similarities to the ATM kinase, which is mutated in radiation-hypersensitive ataxia telangiectasia patients. That the yeast pathway was conserved in eukaryotes was subsequently confirmed by the cloning of mammalian Chk1 kinase homologues (38). Recombinant human Chk1 phosphorylates Cdc25C on Ser216, a state that is maintained throughout the cell cycle except during mitosis (38, 39). The domain containing Ser216 is highly conserved in Cdc25A and Cdc25B. Binding studies revealed that 14-3-3 proteins only associated with Cdc25A, B, or C proteins that were phosphorylated on Ser216.

Consistent with 14-3-3 proteins having a checkpoint function are the observations that rad24 and rad25 encode 14-3-3 proteins and 14-3-3σ is a major p53-induced transcript following irradiation treatment (40, 41). Overexpression of Cdc25C S216A mutant protein, which is incapable of binding to 14-3-3, abrogated an irradiation induced G_2 arrest in HeLa cells, suggesting that 14-3-3 binding is important for inactivating Cdc25 phosphatase activity (39). *In vitro*, 14-3-3 binding causes only a modest suppression of Cdc25 activity (42). This observation led to the proposal that 14-3-3 binding indirectly inhibited Cdc25 function by sequestering Cdc25 in an intracellular location inaccessible to cyclin B1-Cdc2 complexes. Indeed, a recent report has shown that, in fission yeast, 14-3-3 prevents Cdc25 nuclear accumulation in response to DNA damage (43).

Chk2 - Eukaryotic cells do not enter mitosis when DNA replication is blocked, or when DNA is damaged. Candidate genes involved in this S-phase checkpoint response include the related budding yeast Rad53 and fission yeast cds1 kinases (37). Recently, a human Chk2 kinase was isolated based on its sequence similarity to the Rad53/cds1 kinases (44, 45). Analogous to its yeast counterparts, human Chk2 is activated in response to DNA damage and replication blocks, and this activation is ATM dependent. Although Chk2 and Chk1 share little amino acid similarity, Chk2 also phosphorylated Cdc25C on Ser216 *in vitro*.

Such apparently redundant functions with respect to Myt1- and Wee1-mediated phosphorylation of Cdc2 Thr14/Tyr15 and Chk1- and Chk2-mediated phosphorylation of Cdc25C Ser216, complicates the drug discovery process. It is unclear, at least from the published data, whether inhibiting a single checkpoint kinase will be sufficient to abrogate the G_2 DNA damage checkpoint (Figure 1B). It is encouraging, however, that UCN-01 (7-hydroxystaurosporine) is capable of abrogating a DNA damage checkpoint arrest and that this activity correlates directly with Wee1 inactivation and Cdc25C activation (46). Thus, assessing whether UCN-01 inhibits Wee1, Myt1, Chk1, or Chk2 activity may assist in validating one of these checkpoint kinases for further therapeutic development.

REGULATION OF M-PHASE PROGRESSION

In examining potential M-phase therapeutic targets, a description of the transitions within this portion of the cell cycle will provide a useful orientation (see Figure 2). After movement from G_2, the initial mitotic phase is prophase where chromosomes begin to condense and the mitotic spindle begins to form. Prophase progresses into metaphase where the condensed chromosomes are aligned at an equatorial plane attached to the spindle. The transition from metaphase to anaphase occurs as the sister chromatids begin to segregate from the parent chromosomes and migrate to opposite poles of the parent cell. The anaphase transition to telophase is characterized by the disappearance of the mitotic spindle, the reappearance of nucleoli and subsequent formation of the nuclear membrane. Exit from M phase and entry into G_1 phase of the subsequent cell division cycle is accompanied by cytokinesis and the formation of daughter cells.

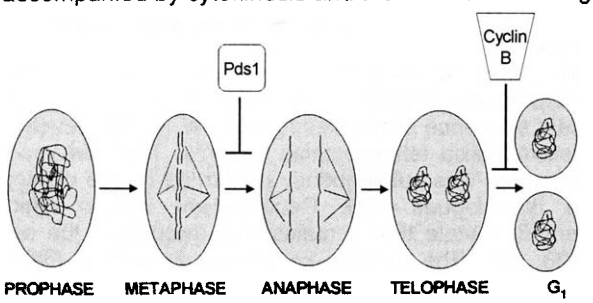

Figure 2. Phases within M phase.

A small portion of the very large number of regulatory events that modulate these steps may be attractive targets for small molecule intervention. Many of the components of these pathways have only been described in yeast systems where the genetics are more tractable to manipulation than in many

higher organisms. Nevertheless, these yeast studies provide useful insights into the regulatory mechanisms and often descriptions of mammalian homologues shortly follow those of the yeast genes.

Ubiquitin-mediated protein degradation - One of the major regulatory events in M phase progression is the directed degradation of components involved in mitotic control. The proteolysis of cyclin B is required, for example, for a cell to successfully exit mitosis and enter the subsequent G_1 phase (47). The degradation of other proteins is required for progression into and through anaphase within M phase. This degradation is controlled by ubiquitination. In the case of cyclin B, targeting depends on a nine amino acid N-terminal sequence on the cyclin called the D (or destruction) box (48, 49). Ubiquitin (Ub) is a 76 amino acid peptide, which is ultimately esterified to the ε-amino group of lysine(s) on the target protein through the action of a complex of Ub transferases (see Figure 3). The initial step in this process requires ATP and results in the formation of a thioester between the C-terminal glycine acyl group of Ub and a cysteine sulfhydryl group on the non-selective Ub activating enzyme (E1). The Ub on the "charged" E1-Ub complex is transferred to a sulfhydryl group on Ub-conjugating enzyme complex (E2). E2 complexes have been identified in yeast as well as mammalian cells. These E2 complexes demonstrate some specificity relative to the ultimate Ub acceptor proteins, but are not cell-cycle regulated. The final catalytic / regulatory component in this pathway is a Ub protein ligase (E3), which can transfer the Ub from E2 to itself and thence to the target protein, or mediate a direct transfer from E2 to the target (50). Once a protein is singly ubiquitinated, additional Ub is added to lysines on the initial Ub moiety to form poly-Ub chains. Target proteins Ub-tagged in this fashion are then subject to degradation by 26S proteosomes.

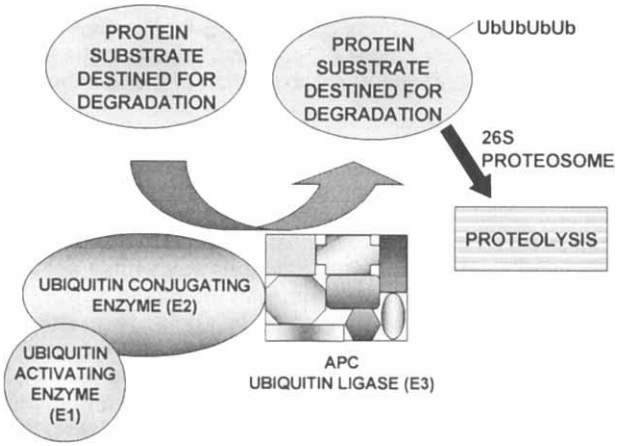

Figure 3. Ubiquitin mediated proteolysis pathway

Among the ubiquitination pathways involved in cell cycle control, perhaps the greatest potential therapeutic interventions may be found in the E2 Ub conjugating enzymes and the E3 Ub ligase complex, more specifically the anaphase promoting complex (APC), since the activities of these two components (E2 and E3) appear to be cell cycle specific.

M phase specific Ub-conjugating enzymes - Cyclin B specific E2 components have been described in a number of organisms from yeast to *Xenopus* to humans, (47, 51-53). One of these Ub conjugating components with substrate selectivity for cyclin B is called UBCx and has been isolated from *Xenopus* nuclear extracts (52). The Ub conjugating activity was dependent on the presence of the intact D box sequence of sea urchin cyclin B used as substrate in an *in vitro* ubiquitination assay. A second E2 enzyme called hUBC9 has substantial sequence homology to a yeast E2 called ScUBC9 that possesses some selectivity for yeast cyclin B homologues (51). In yeast the human protein complements the function of a mutant yeast ScUBC9, resulting in the accumulation of yeast cyclin B homologues and lack of progression

through M phase. These data suggest that hUBC9 is functionally related to the yeast ScUBC9. A third E2 enzyme with cyclin substrate specificity called UbcH10 has also been reported (53). This human protein can functionally substitute for an authentic clam E2 in an *in vitro* clam assay system. In addition, a dominant negative mutant of this protein made by changing the active site cysteine (a common feature in all known E2 proteins) to serine, resulted in inhibition of destruction of cyclin B, and when introduced into cycling cells, caused an accumulation of cells in mitosis prior to anaphase. The existence of a small number of enzymes (all with an analogous active site cysteine) with an apparent substrate preference for D box containing B-type cyclins point to a possible mechanistic target with some selectivity, assuming that the UBC/cyclin B protein-protein interactions might be specifically inhibited by an appropriate small molecule. That is, E2 proteins that have a common class of preferred substrates (D box containing B-type cyclins) may be a target that is able to afford selectivity over other E2 proteins. Whether this is an achievable goal may depend on a more complete characterization of these initial E2 Ub conjugating enzymes.

M phase specific Ub ligase or APC - The M phase specific E3 complex APC, which is both necessary and sufficient for substrate protein ubiquitination in the presence of a Ub-loaded E2, is comprised of at least eight protein subunits that provide E2/substrate selectivity interactions as well as APC regulation (47, 49, 50). APC is the only component of the ubiquitination/protein degradation pathway whose activity appears to be regulated in a cell cycle dependent fashion with increased activity in M phase dependent on Cdk and perhaps mediated by polo like kinase phosphorylation. Since at this point the intra-APC interactions of the eight components are not yet well characterized, the opportunity for direct modulation of APC seems limited. However, some of the substrates for APC are known and these substrates may represent useful targets. Additionally, those pathways regulating the APC substrates may be potential sites of therapeutic intervention as well.

APC substrates - One substrate of the APC of budding yeast is a protein called Pds1 that inhibits anaphase progression by interacting with sister chromatids and keeping them from separating from one another (54-56). This protein, or more interestingly, the kinase that phosphorylates it in response to DNA damage, may be an attractive target since the phosphorylated form of Pds1 may be more resistant to the ubiquitination catalyzed by APC and is required for release of inhibition of anaphase progression.

Regulation of APC - The opportunity for modulation of catalytic activity affecting APC action is limited at this point since these pathways are only beginning to be described. What is known from work in yeast, *Xenopus*, and mammals is that at least two regulatory proteins are able to cause M phase activation of APC (57-64). The first protein (or related proteins) is called variously CDC20, Fizzy, or p55Cdc and its binding to APC is required for ubiquitination of Pds1 and subsequent anaphase progression. A second protein whose binding to APC is required for degradation of B-type cyclins and exit from telophase and mitosis is called Cdh1. The levels of CDC20 are normally greatest at mitosis and are relatively low in other phases of the cell cycle whereas Cdh1 levels appear to be constant (65). When the mechanisms that control the levels of CDC20 and Cdh1 are more completely described, perhaps some entry into pharmacologic modulation may be available. At this point, the only indication of such an opportunity comes from the observation that immunoprecipitated complexes p55Cdc exhibited elevated kinase activity in cycling cells compared to quiescent cells (62).

A protein or family of proteins that interacts with CDC20 to regulate its activation of APC is being characterized (66-70). The prototypical member of this family is called Mad2 (for mitotic arrest deficient) and it functions in a mitotic spindle checkpoint that monitors the attachment of microtubules to kinetochore structures

on the centromere of replicating chromosomes at the metaphase-anaphase transition prior to the movement of the separating chromatids along the mitotic spindle. Mad2 binds to the kinetochore of an improperly unattached chromatid and blocks progression of anaphase until the chromatid pair is attached to opposite poles of the spindle and distributed into the daughter cells upon completion of mitosis. The mechanism of Mad2 inhibition of CDC20 activation of the APC and subsequent ubiquitination and proteolysis of Pds1 is not clear but may depend on oligimerization of Mad2 monomers or on interaction of other members of the Mad family, Mad1 and Mad3. Another analogous set of CDC20 modulators called Bub exist in yeast. Genetic defects in Mad2 lead to aberrantly distributed chromosomes suggesting that interfering with its function may bypass the checkpoint with presumably negative consequences for cell survival. Whether phosphorylation plays a role in regulating either Mad or CDC20 activity is an open question. Some evidence exists that phosphorylation of one or more of the components of the APC (either directly or mediated or modulated in some fashion by regulatory proteins) may influence the activity of the complex (71).

Although much remains to fully describe the regulated pathway of mitosis-dependent protein degradation, many of the components that participate in this process have been described, some possessing catalytic ubiquitination or phosphorylation activity (or regulated by such action), and thus may provide exploitable targets for therapeutic intervention.

ONCOGENIC G_2- AND M-PHASE KINASES

Aurora kinases – In mammalian cells, a family of related serine/threonine kinases have been recently identified that share structural and functional similarities to the *Drosophila* aurora kinase. Most notable, one of these family members, aurora2 (also known as STK15 and BTAK), is a potential oncogene since it is amplified and overexpressed in many breast and colon tumor cells (72, 73). Additionally, aurora2 overexpression transformed NIH 3T3 cells and these transformed cells formed tumors in nude mice. The *Drosophila* aurora gene was originally identified as a mutation in which the centrosome duplicated but failed to separate and form a bipolar spindle (74). In many cases, these closely paired centrosomes formed a monopolar spindle, which subsequently led to embryos containing mitotic abnormalities such as polyploid nuclei. Consistent with a role in centrosome separation and chromosome segregation, human aurora2 was localized to the centrosome, spindle poles and the spindle during metaphase and anaphase. Additionally, transient overexpression of aurora2 results in a significant increase in centrosome copy number and aneuploidy. Human aurora1 (also known as AIM1), which shares 74% amino acid identity with aurora2 within the kinase domain, was localized to a distinct subset of mitotic structures, for example the midzone and telophase disc during the anaphase to telophase transition, suggesting that it performs distinct mitotic function(s). Aurora1 expression levels are also high in many tumor cell lines but, in contrast to aurora2, aurora1 overexpression has been postulated to function in a dominant-negative manner to cause mitotic abnormalities (75). A third mammalian aurora-related kinase, STK13, has been reported recently, but its potential mitotic function has not been characterized (76).

Polo-like kinases – The Polo-like kinases (Plks) are a family of serine/threonine kinases structurally related to polo in *Drosophila* (for review, see 77). Like aurora, *Drosophila* polo mutants display spindle abnormalities (78). The plks share a conserved amino-terminal catalytic domain and a highly conserved sequence motif (the polo-box) in the carboxyl termini. Mammals contain at least three plks of which Plk1 is most similar to *Drosophila* polo. The other two human plks (termed Snk and Prk) were identified as immediate early genes after growth stimulation and thus likely play a role during interphase. Plk1 mRNA expression is frequently elevated in non-small cell lung cancer (79). Furthermore, overexpression of Plk1 transforms

NIH-3T3 cells and these transformed cells formed tumors in nude mice (80). Plk1 function is complex and appears to regulate multiple stages of mitosis; it has a role in regulating Cdc25C activation during mitotic initiation, controlling the APC complex during the metaphase to anaphase transition, and in execution of cytokinesis. Of potential therapeutic relevance, reduction of Plk1 activity by microinjection of anti-Plk1 neutralizing antibodies induced dramatic aberrant mitotic events in HeLa cells but, in contrast, nonimmortalized Hs68 cells arrested as single, mononucleated cells in G_2 (81). Thus, inhibition of Plk1 activity may induce a spindle checkpoint response leading to reduced viability in tumor cells that are either defective in this checkpoint response.

SUMMARY

The central role that Cdk-Cyclins complexes play in controlling cell cycle progression has generated much interest as a collection of potential targets for the therapeutic intervention in proliferative disorders. Many Cdk inhibitors have been developed that effectively block cell division in preclinical settings and some of these are currently in clinical trials. Rapid advancements in identifying other key players that regulate cell cycle events during G_2 to M phase transitions have also occurred during the past five years. Some of these events include the G_2 DNA damage response, centrosome/spindle assembly, and anaphase-promoting complex pathways. These pathways are modulated by different protein kinases, thus many potential targets for the discovery of small molecule inhibitors. However, as is the case for the Cdks, many of these kinases are members of highly homologous families so compound specificity will present a challenge to the medicinal chemist. Development of new research tools is needed to overcome these challenges. Other discoveries in biology might reveal novel regulatory mechanisms and uncover other opportunities for drug development.

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Chapter 26. New Targets in Autoimmune Diseases

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Nature has provided in the phagocytes . . . a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all diseases and that is to stimulate the phagocytes.

G.B.Shaw. The Doctor's Dilemma

In the early twentieth century, the ideas of Metchnikoff generated sufficient fierce public debate to stimulate the writing of this play. However, few now doubt the value of directing the power of the immune system through specific immunization to fight infections. The idea of exploiting the immune system for therapeutic intervention (immuno-therapy) is one that is expanding and is currently the focus of research efforts for developing novel treatments in a number of diseases.

Macrophages or phagocytes are known to play an essential role in clearing the body of virus-infected cells through targeted cell killing. However, as was recognized in this play, there is a very fine balance between the need to stimulate the immune system to destroy pathogens and the need to control the system to prevent destruction of healthy tissues. When the immune system is misdirected against apparently normal tissue, the resulting damage leads to auto-immune diseases such as rheumatoid arthritis, type I diabetes mellitus, idiopathic thrombocytopenia and glomerulonephritides which all are characterized by pathological tissue destruction. Later in this play, Shaw addresses the pivotal role of antibodies (opsonins) in stimulating phagocytes and even proposes the basis of auto-immunity.

How antibody-mediated opsonisation of pathogens led to phagocyte activation took a long time to unravel, but it is clear now that surface receptors on a variety of immune cells define the nature of the cellular response to immune complexes (1-4). Expression of these receptors is tightly regulated in terms of cell type and number. The purpose of this review is to provide an overview of the role of these receptors in the immune response to opsonised antigen as revealed by the use of targeted gene knock-out technology. Understanding the mechanisms that control the activation of macrophages and natural killer cells offers new approaches to either modulate the immune response for treating antibody mediated diseases or to kill malignant cells and virus infected cells. Furthermore, modulation of the inhibitory signals that abort activation pathways can prevent targeted cell killing and tissue destruction. Thus the ability to control activation and inhibition signals offers novel opportunities for drug discovery.

IMMUNOGLOBULINS

The diversity of antibodies that recognize a wide variety of different antigens results from somatic recombination events which alter the variable region or F(ab) of the antibody (Figure 1). Through this recombination process, the immune system

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generates the enormous diversity and flexibility required to protect the host against all foreign antigens encountered during life. However, in the vast majority of cases, antibody recognition of antigen alone is insufficient to provide protection. The major exception to this are the "neutralising" antibodies where F(ab) binding masks an epitope on the pathogen critical for its function, such as the protein coat of a virus required for viral infection of cells.

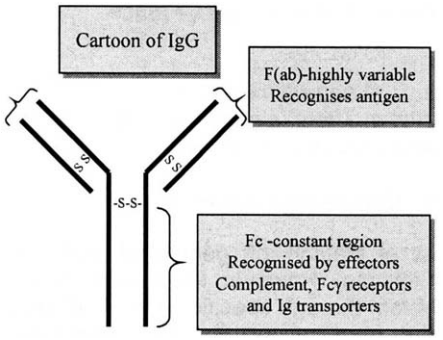

Figure 1. Immunoglobulins

The remainder of the antibody molecule consists of the Fc portion (Fc = fragment crystalline or constant), which is conserved. Antibodies are subdivided into classes depending on the Fc portion of the molecule. There are five major classes of antibodies or immunoglobulins (Ig): IgG, IgM, IgA, IgE and IgD. The most abundant antibody in plasma is IgG. Within each major class there are, in addition, antibody subtypes. For IgG in man, there are four subtypes IgG1, IgG2, IgG3 and IgG4. The predominant circulating antibody in man is IgG1. Antibodies circulate as monomers but are clustered to form immune complexes by antigen.

Antibodies are recognized by three types of effector molecules: complement, Fc receptors and transporter receptors (1-7). The complement cascade can be activated by immune complexes. However, studies in complement deficient mice have shown surprisingly that these animals have a normal cytotoxic response suggesting that immunal complex mediated diseases may be attributed to other factors (8).

IMMUNOGLOBULIN RECEPTORS (FcRs)

The Fc regions differ for each class of antibody, and these are recognized by a specific set of Fc receptors. The immunoglobulin class recognized by each receptor class is specified by a Greek letter. Thus, IgG is recognized by Fcγ receptors (FcγRs), IgA by Fcα receptors, IgM by Fcμ receptors and IgE by Fcε receptors. Several of these receptors constitute families of proteins which display different cell distribution, specificity and affinity for their respective ligands. For example, IgE is recognized by a high affinity receptor, FcεRI, and a low affinity receptor, CD23. These two receptors have distinct distributions and roles in regulating the immune response to IgE. The Fcγ receptors that recognize IgG comprise a complex family of proteins encoded by at least 8 genes in man, all clustered on chromosome 1.

Fc receptors have a defined cellular location and this dictates the nature of the cellular response to antigen-antibody complexes. The high affinity Fcε receptor is found on mast cells and basophils (9). Aggregating the receptor with IgE complexed to allergan results in degranulation of the mast cell with the release of histamine and the typical "allergic" response. If the IgE was unable to dock to a receptor, the host would not be able to mount an immune response to the challenge. Fc receptors play the pivotal role in coordinating the immune response to antigen by linking the cellular and humoral (antibody) responses. Recent work using targeted gene knock-out technology has demonstrated the central role that these receptors play in regulating immune cell function and in mediating cytotoxicity and auto-immune disease (8).

Fcγ RECEPTOR FAMILY

Three classes of Fcγ receptor have been identified based on their affinity for IgG and recognition by monoclonal antibodies. Thus, FcγRI (CD64) is a high affinity receptor that recognizes monomeric IgG (10). Surface expression is tightly regulated by interferon-γ (IFNγ). FcγRI is found on macrophages, monocytes and IFNγ stimulated neutrophils. There are three genes for FcγRI, all located on the same region of chromosome 1. However, only one of these genes encodes the transmembrane anchored receptor. Stop codons in the coding sequences in the extracellular regions of the other two genes predict truncated soluble proteins (11).

FcγRII (CD32) is a low affinity receptor that is widely distributed (12). There are three genes encoding FcγRII; FcγRIIa, FcγRIIb and FcγRIIc (13). FcγRIIb is the dominant negative regulator of immune responses to antibodies.

FcγRIII (CD16) is also a low affinity receptor. There are two genes in humans, but only one in mouse. In humans, the two genes are highly conserved but the nucleotide differences encode changes that radically alter the post-translational processing of the two genes (14). FcγRIIIa is anchored to the cell surface through a transmembrane domain whereas FcγRIIIb is anchored via a glycosyl phosphatidylinositol (GPI) anchor. FcγRIIIa is found on natural killer cells, macrophages and mast cells whereas FcγRIIIb is found only on neutrophils.

With the exception of FcγRIIIb which is a GPI anchored protein, all other FcγR receptors are type I integral membrane proteins comprising an amino terminal extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tail. The extracellular region comprises protein modules of the immunoglobulin superfamily (IgSF) type which are variously described as C2-like, V-like or I-like in nature (2, 3). Each IgSF is encoded by a separate exon and all the Fcγ receptors have two of these IgSFs except FcγRI which has three. The genes for these receptors are found on chromosome 1 and the overall similarity in genomic organization suggests that receptor diversity has likely arisen as a result of exon duplication and shuffling (15). By contrast, the cytoplasmic tails of FcγR are highly diverse and potentially define different signalling pathways.

Among the cytoplasmic domains, only the FcγRII genes encode recognizable motifs that trigger signalling cascades. The three isoforms of FcγRII differ in the nature of their cytoplasmic tails, implying the recruitment of different signalling pathways (1-4,13). FcγRIIa has an ITAM (immunoreceptor tyrosine activation motif) motif that typically consists of D/E $(X)_2$ YXXL $(X)_7$ YXXL where X = any amino acid (16-18). The ITAM for FcγRIIa is unusual and has 11 amino acids instead of the conventional 7 amino acids that separate the YXXL motifs (12,16). As the intervening amino acids $(X)_7$ are thought to specify the Src family kinase that is recruited, this suggests that FcγRIIa may recruit another kinase (17). ITAM association is required for tyrosine kinase activation (16-18) (Figure 2). Thus, following aggregation of ITAMs, Src-like kinases phosphorylate the two tyrosine residues. In myeloid cells, the preferred Src-kinase for many ITAMs is Lyn kinase, however, the kinase preference for FcγRIIa is not yet known. Once phosphorylated, the tyrosine residues act as high affinity binding sites for Syk kinase which translocates from the cytoplasm to the plasma membrane and activated Syk kinase initiates the signal transduction cascades leading to cell activation. Studies in the Syk knock-out mice indicate that FcγRIIa requires intact Syk kinase to mediate signalling events (19).

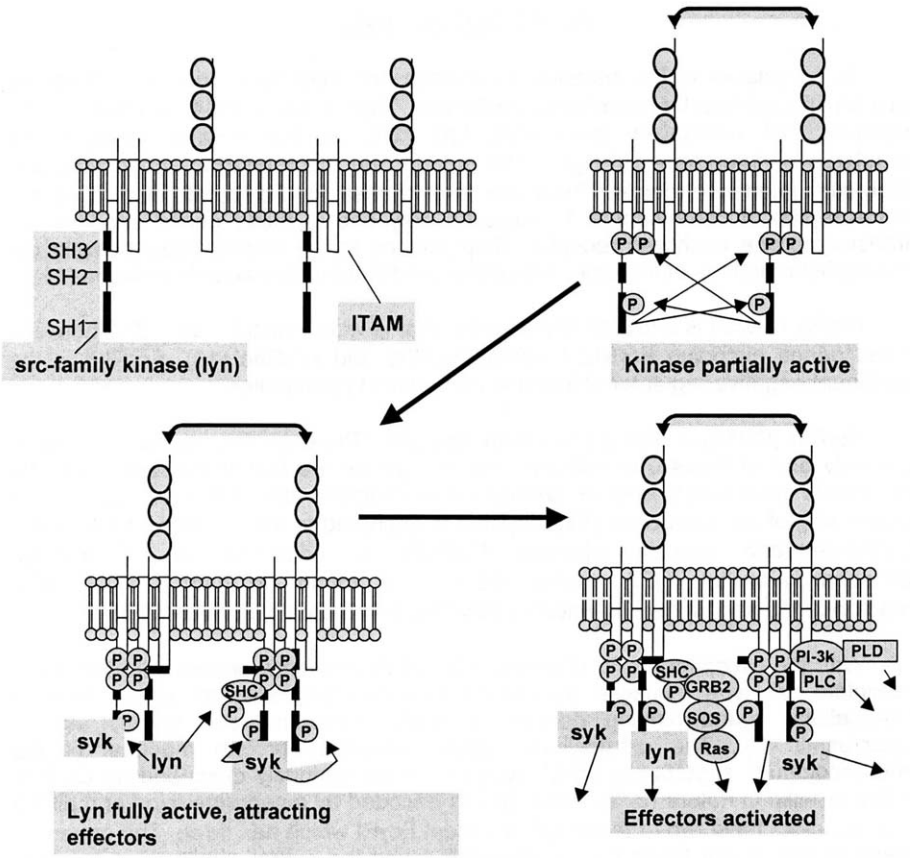

Figure 2. Model for Cell activation signalling: FcR aggregation by immune complex

The cytoplasmic tail of FcγRIIb contains an ITIM (immunoreceptor tyrosine inhibitory motif) (20). The tyrosine based ITAM and ITIM motifs regulate activation or inhibitory signals within the cell. Recent evidence indicates that the ITIM motif on FcγRIIb is critical for aborting the activation signals generated through FcγRI on macrophages, the B cell receptor on B lymphocytes and FcεRI on mast cells (21-24) (Figure 3). Some ITIMs recruit tyrosine phosphatases (25,26), while others such as the ITIM on FcγRIIb recruit the inositol phosphatase SHIP (SH2 containing inositol phosphatase) (27,28). The principal substrate for SHIP is phosphatidyl inositol phosphate (PIP3) (3-5) and it inactivates the key signalling molecule in the activation cascade by removing the 5' phosphate from the inositol ring (27).Thus, FcγRIIa and FcγRIIb elicit opposing functional effects by recruiting a kinase or a phosphatase through the respective ITAM or ITIM.

The cytoplasmic tails of FcγRI and FcγRIII bear no known signalling motifs; indeed, for GPI anchored FcγRIIIb, there is no typical cytoplasmic tail (10,14). Yet aggregation of any of these receptors results in the initiation of signal transduction cascades that are characterized by tyrosine kinase activation through the sequential recruitment and activation of Src family kinases (usually Lyn kinase) and Syk activation (29-32). Recent data has shown that these receptors function through the recruitment of an accessory molecule known as the γ chain (33-36).

THE γ CHAIN: AN ACCESSORY MOLECULE FOR Fc RECEPTOR SIGNALLING

The γ chain is a member of a family of signal transducing molecules which includes the ζ chain of the T cell receptor (36). The γ chain is a molecule of 7kDa that resembles a plasma membrane anchored ITAM (35). It has a short extracellular region of only 4 amino acids, one of which is a cysteine residue that allows the formation of homodimers. There is an unusual transmembrane domain with an aspartate residue predicted to lie on the first turn of the α helix. The cytoplasmic domain contains a classical ITAM – D/E $(X)_2$ YXXL $(X)_7$ YXXL, that is responsible for initiating signal transduction events through tyrosine kinase activation (37, 38). The tyrosine residues in the ITAM are phosphorylated by Src family kinases (usually Lyn) and this phosphorylated ITAM is also a high affinity recognition site for Syk. Gene knock-out studies have shown that both the γ chain and Syk are essential for cell activation by immune complexes through Fc receptor aggregation (19). In natural killer cells where the ζ chain is also expressed, homodimers of ζ and γ chains and ζγ heterodimers have been demonstrated, and FcγRIII can associate with any of these (39, 40).

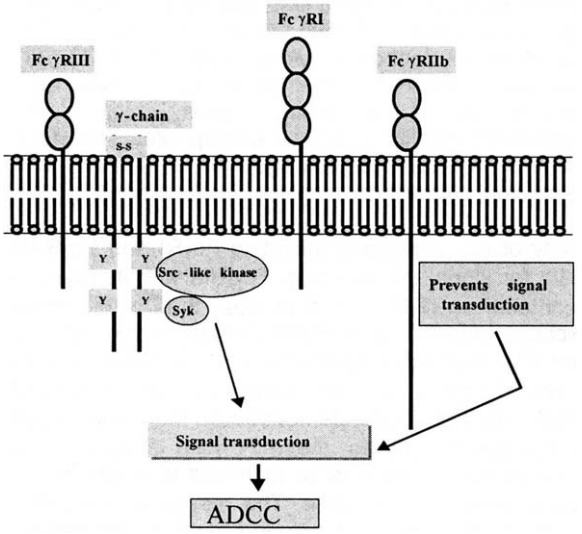

Figure 3. Fcγ Receptors

Other Fc receptors that use the γ chain for signal transduction include the high affinity IgE receptor, FcεRI and the IgA receptor, FcαRI. The γ chain may associate with each of the various receptors through the respective transmembrane domains, with the aspartate residue perhaps playing an important role. The thermodynamics of including a charged residue in the lipid environment of the membrane is highly unfavorable and their presence often implies the presence of an interacting residue that is capable of forming an ion pair. However, only the transmembrane domain of FcαRI contains a residue capable of forming this ion pair interaction with the γ chain aspartate. The mechanism of association between the γ chain and the other receptors (FcγRI, FcεRI, FcγRIII) remains undefined although it is clear that aggregation of the ligand binding chain by immune complexes on the cell surface results in clustering of intracellular γ chain and initiation of signal transduction. The γ chain can be limiting in cells that express several receptor types that compete for the same signal transducing molecule. Thus, the extracellular ligand must provide the selective initiating event through occupancy of the appropriate receptor.

THE BALANCE BETWEEN ACTIVATION AND INHIBITION

The γ chain contains a classical ITAM. Aggregation of the binding chain (FcγRI, FcγIIIa, FcαRI and FcεRI) by immune complexes results in clustering of the γ chain, phosphorylation of the γ chain tyrosine residues and activation of kinases to initiate signal transduction. Data obtained from the γ chain deficient mice indicates the

central role of this process in antibody dependent cytotoxicity. The low affinity FcγRIIb on B cells contains the inhibitory ITIM motif and plays a critical role in inhibiting B cell proliferation through the B cell receptor (21, 22). Co-ligation of the B cell receptor with FcγRIIb results in an aborted B cell response (49). Similarly, mast cell activation can be prevented by recruiting FcγRIIb (23). The down-regulatory role of FcγRIIb in B lymphocytes and mast cells has been confirmed using FcγRIIb deficient mice whose phenotype of augmented humoral and anaphylactic responses provide evidence for unrestricted B lymphocyte and mast cell activity (50). The ITIM of FcγRIIb recruits the enzyme SHIP which dephosphorylates PtdIns(3,4,5)P3 (28). This intracellular signalling molecule is required in the activation cascade, as it binds to the pleckstrin homology domain of Tec family kinases, which in turn activates phospholipase C to generate inositol (1,4,5) trisphosphate. Therefore, dephosphorylation of PtdIns(3,4,5)P3 prevents the prolonged generation of InsP3 which is necessary for sustained calcium signalling.

Macrophages display a variety of Fcγ receptors, including FcγRI and FcγRIIIa which use the ITAM of the γ chain for signal transduction, FcγRIIa with its unusual ITAM (12) and FcγRIIb which contains an ITIM (20). It is clear that, within these cells, intracellular signalling cascades induced by immune complexes depends on the relative amounts of ITAM and ITIM signalling molecules. FcγRIIb deficient mice were also used to explore the role of FcγRIIb on macrophages using a model of immune complex induced alveolitis (51). This model demonstrated that, when given subthreshold concentrations of immune complexes, these FcγRIIb deficient mice developed a robust inflammatory response in contrast to their wild type littermates where the same challenge failed to elicit any response. The response in FcγRIIb deficient mice was characteristic of inflammation, and included hemorrhage, edema and neutrophil infiltration. The higher concentrations of tumour necrosis factor and chemotactic factors in the bronchiolar lavage fluid demonstrated a role of the resident lung macrophages in mediating this disease; thereby confirming the negative regulation of FcγRIIb on macrophage function.

Thus, the response of macrophages can be fine tuned by balancing the relative input from ITAMs and ITIMs and clearly it may be important to understand the mechanisms regulating this balance. However, additional levels of control come into play as the γ chain may in a number of cases be limiting and different receptors compete for the same signal transducing molecule. The implications of this are likely important as it opens the possibility of allowing receptors to couple to different effector molecules depending on the differentiation state of the cell and the relative proportions of other Fc receptors on the cell surface. Such a switch in coupling has been demonstrated recently for the high affinity Fc receptor, FcγRI, using a cell line model of differentiation (52).

In human cells, both FcγRIIIa and FcεRI require the presence of γ chain for cell surface expression. Newly synthesized FcγRIIIa and FcεRI peptides contain lysosomal degradation signals and are directed to the lysosomes in the absence of the γ chain. These two receptors are frequently found on the same cell type such as mast cells. Thus, the amount of the γ chain limits the surface expression of both receptors. Abrogation of one receptor by gene knock-out (e.g. FcεRI) leads to an increase in expression of the other (FcγRIIIa). A functional consequence, surprisingly, is that degranulation of mast cells in response to ovalbumin challenge is enhanced in the FcεRI knock-out mouse (41). These FcεRI deficient mice displayed an exaggerated anaphylactic response due to increased surface expression of FcγRIIIa (42). This data suggests that by limiting the amounts of γ chain and thus the surface receptor expression, the immune system has an alternative response to antigen challenge.

γ CHAIN AND Fc RECEPTORS IN DISEASE

Auto-immunity is often characterized by pathological tissue damage initiated by immune complexes between antibodies and other antigens. These can mediate damage to tissues through: 1) mechanical plugging by immune complexes in small capillaries (especially the glomerulus), 2) activation of complement, and 3) activation of Fc receptors bearing cells. Mice that are deficient in complement or Fc receptors have helped elucidate the pathophysiological mechanisms of immune complex damage. Similarly, the γ chain deficient mice have revealed that this molecule is critical in regulating immune complex mediated cytotoxicity.

Initial studies examined the Arthus reaction, a local inflammatory response in the skin characterized by edema, haemorrhage and neutrophil infiltration following the deposition of immune complexes in the skin after injection of antigen. In this model of hypersensitivity, the response was completely attenuated in γ chain deficient mice (43, 44). However, in complement deficient mice the response was identical to the wild-type mouse. This finding was unexpected, as the Arthus reaction has typically been attributed to complement activation. Dependence on mast cell FcγRIIIa was confirmed when the Arthus reaction was absent in mast cell deficient mice but could be restored by mast cells from wild type mice but not from γ chain or FcγRIIIa deficient mice (45).

Additional studies have examined the role of the γ chain in mediating the progression of immune complex glomerulonephritis. The γ chain deficient mice were crossed with the NZB/NZW strain, a well-characterized strain that develops spontaneous nephritis (46). Mice heterozygous for the γ chain displayed disease progression identical to that observed for the parental strain. However, mice deficient in the γ chain had no histological evidence for glomerulonephritis and no proteinuria despite the presence of autoantibodies, circulating immune complexes and deposition of immune complexes and complement in the glomeruli (47). Thus the cytotoxicity associated with immune complex disease requires the γ chain and probably intact FcR responses.

Targeted cell killing is necessary to clear the body of cancer cells and virus infected cells. The central role of the γ chain in mediating antibody dependent cytotoxicity was confirmed when the γ chain deficient mice were unable to mount a cytotoxic response *in vivo* using an experimental model of melanoma (48). Melanoma cells express an antigen on the cell surface as a product of the brown locus (protein gp75). Antibodies to this protein introduced by administering a monoclonal antibody or through active immunization are effective at preventing or eradicating lung melanoma metastases. However, mice deficient in the γ chain had lost this protection despite normal levels of anti-gp75 antibodies, thereby, implicating the critical role for Fcγ receptors in tumor cytotoxicity.

CONCLUSION

Receptors for immunoglobulins play a pivotal role in linking the cellular and humoral arms of the immune system. These receptors are required to initiate the cellular immune response to antibody/antigen complexes. They are a complex gene family of proteins and are tightly regulated in their cellular expression. These receptors are implicated in various disease states including antibody driven autoimmunity and allergic conditions. Recent studies using targeted gene knock-out technology has provided clear-cut evidence for the role of these receptors in mediating antibody dependent cytotoxicity which can be either protective or destructive. In addition, Fc receptors mediate a fine balance between activation and inhibition of activation status of a variety of cells including B and T lymphocytes, mast cells and macrophages. Understanding the mechanisms that underly the action of these receptors provides a novel approach to harness their activity to treat a variety of diseases including cancer, persistent viral infections, auto-immunity and allergy.

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SECTION VI. TOPICS IN DRUG DISCOVERY AND DESIGN

Editor: George L. Trainor, The DuPont Pharmaceutical Company, Wilmington, Delaware

Chapter 27. Application of Combinatorial and Parallel Synthesis to Medicinal Chemistry

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Introduction - In past years, a wide diversity of technologies has been used in the practice of combinatorial chemistry, despite the commonly shared goal of accelerating the discovery of drugs and materials through rapid synthesis of new compounds. However, consensus has recently begun to evolve in the application of these technologies. For example, library quality (as defined by performance in screening) is now understood to be an important part of the success of any diversity program. A library with a high false-positive rate can consume significant chemistry and biology resources in futile hit confirmation attempts. Without good hit confirmation, a false lead may be sent on for further optimization, resulting in even greater loss of time, effort, and ultimately, credibility. For this reason, full chemistry development and validation are now generally recognized as critical in the practice of combinatorial chemistry, despite the additional effort entailed.

There is also a growing consensus on the use of single compounds over mixtures, although some notable exceptions exist. Further, researchers who once were primarily committed to increasing chemical diversity are often becoming more concerned with directing the structures in their libraries. Conversely, programs that formerly involved only "rationally designed" libraries are often incorporating more empirical, diversity-based approaches to lead generation. Companies based on a unique technology are diversifying to include some of the simpler, more traditional capabilities. And those focussed on traditional approaches are adding more high-tech components into their programs, such as automated high-throughput purification. The same can be said for the use of solid- versus solution-phase synthesis. Most practitioners now use a combination of both methods, often in the same library synthesis.

The special importance of organizational and control systems in combinatorial chemistry is also now recognized to be greater than in most other research endeavors, particularly for large, diversity-based programs. The shepherding of thousands of compounds through a synthetic sequence without error requires well-defined systems to describe the flow of materials and information within the organization.

This report covers recent advances reported in the rapidly expanding literature of combinatorial chemistry. The first half, covering methodology, has been loosely arranged according to the usual sequence of steps in building a library, from design through quality control. The second half summarizes recent results in the application of combinatorial chemistry to drug discovery, first as applied to lead generation, then for lead optimization.

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CONSIDERATIONS FOR LIBRARY DESIGN AND IMPLEMENTATION

Scaffold and precursor selection - An increasing emphasis has been placed on making libraries of "drug-like" molecules in order to increase the likelihood of finding a new lead or optimizing a known active structure which possesses improved pharmacological parameters, for example, *in vivo* solubility and permeability. Recently, Lipinski described experimental and computational approaches to the analysis of molecular factors that influence these pharmacological parameters (1). Dubbed "the rule of 5," Lipinski proposed that molecules possessing one or more of the following properties are likely to exhibit poor absorption or permeability: more than 5 H-bond donors, 10 H-bond acceptors, molecular weights greater than 500 and ClogP values greater than 5 (2). Furthermore, drug leads identified from HTS of combinatorial libraries have been shown to be generally less soluble than drugs screened before HTS and library synthesis was common. The use of "the rule of 5" for library design has become a primary guiding force in many combinatorial efforts. While the historical evolution of combinatorial chemistry has been associated with peptide synthesis, peptides as a structural class have been shunned due to their poor pharmacological properties, some of which are predicted by Lipinski's rules. While peptoids (N-substituted glycines) were created to overcome some of the *in vivo* limitations of peptides, for example enzymatic amide bond cleavage (3), the current focus for most researchers in the field is the generation of libraries based on small molecules (4).

Precursor or building block selection - Molecular diversity has become a crucial factor in designing libraries to identify new lead structures for biological targets, especially in cases where no lead or endogenous inhibitor is known. Often screening libraries attempt to maximize the diversity space covered by the side-chain and/or building blocks employed to ensure that redundancy by similar precursors is minimal, in order to cover enough diversity space to increase the probability of finding hits for a variety of targets. Recently, many computer algorithms have been described for this purpose. One of the earliest approaches to diversity involves generating descriptors that quantify parameters such as lipophilicity, shape, branching, chemical functionality and specific binding features (5). Ultimately these parameters could be employed to select sets of diverse side-chains. A more recent computational approach to molecular diversity involves the use of a flexible surface-based system calculated from a spanning tree analysis of an entire set of molecules, relative to a pair of molecules' dissimilarities (6). These dissimilarities are first calculated taking into account their conformational flexibilities. Other publications have addressed diversity from the standpoint of pharmacological parameters, similar to Lipinski's "rule of 5," through analysis of structural features found in marketed drugs and the Comprehensive Medicinal Chemistry database (7-13). For example, analysis of this database indicated that functionality such as tertiary aliphatic amines, hydroxyl groups, and carboxamides are most abundant in drugs represented (8). While one can mine databases for guidance on side-chain selection, often practical issues, such as synthetic accessibility or solubility, or even cost (assuming the precursor can be purchased), decide whether or not a drug-like or diverse building block is ultimately incorporated in a library.

Library Purification - While most synthetic and medicinal chemists have been accustomed to purifying compounds one at a time by column chromatography, preparative thin-layer chromatography (TLC), reverse-phase HPLC or recrystallization, these techniques become less practical when one has to consider the purification of a large number of compounds. Various solutions to this problem have been described, in addition to the many advances in parallel purification based on the methods mentioned above. Some techniques for purification in a manner that can be incorporated into library generation have been published and are discussed below

(14,15). Many of these purification methods have been developed for solution-phase synthesis, since excess or unreacted reagents will be contaminants in the reaction mixture. The strength of solid-phase synthesis has always been the fact that excess reagents can be employed and unreacted reactants can be washed from the resin matrix.

One limitation, particularly for solution-phase library synthesis, is the ability to perform simple liquid-liquid extractions. An aqueous workup is one of the most common processes in solution-phase synthesis, and traditional methods for this become much more complicated to apply in a parallel format. While some reports describing automated phase separation based on measured volumes have appeared in the literature, this method has can be slow and inefficient, especially when a drying step is required (16). A more novel approach employs the capture of the frozen aqueous phase of an extraction mixture on a set of pins when cooled to –20°C (17). Parallel liquid-liquid extraction with a stationary phase designed to absorb the aqueous phase along with water-soluble impurities after the aqueous and organic phases are allowed to interact has also been described (13). This concept was borrowed from drug and pesticide analysis procedures and is referred to solid-supported liquid-liquid extraction (SLE), and has been automated (16,18,19). Another development, the "fluorous-phase" approach to purification takes advantage of the fact that perfluorohydrocarbons possess poor solubility in organic solvents (20-23). By employing reagents that contain a perfluorohydrocarbon tag, these reagents and their byproducts can be removed by their preferential solubility properties in fluorous solvents, relative to organic or aqueous phases.

Resin-mediated purification methods have also been exploited for solution-phase reactions as well as high-throughput purification (24,25). Ion-exchange resins have been employed for this purpose for many years and these have been extended to parallel purification as well. A recent example of the parallel synthesis of amides demonstrated that these resins could circumvent the use of traditional organic bases that would have to be removed by liquid-liquid extraction (26). Ion-exchange resins have also been used for both chemical reactions and purifications. Use of ion exchange and other polymeric resins as traps for reaction products and intermediates have been employed as a method of purification. For example, BOC-protected amines can be deprotected with a sulfonic acid resin and the resulting resin-bound amine salts separated from impurities by simply washing the resin. The amine product is then isolated from the resin with ammonia in methanol (27). The term "resin-capture" has been applied to such transformations, and employed with polystyrene resins to purify and further functionalize the reaction products of several solution-phase syntheses (28,29). Several groups have exploited functionalized polystyrene resins for removal of excess reactive species (acid halides, isocyanates and sulfonyl chlorides), as well as other reagents (e.g. amines) that may complicate analysis or library screening (30-32). Processes employing these "scavenger" resins have been automated and applied to lead optimization studies (*vide infra*). A multistep synthesis of a complex heterocycle was performed in one-pot with three resin-based reagents, taking advantage of the fact that the reactive functionalities of different resins do not interact (33). The desired product was obtained in 48% yield after filtration of the polymeric reagents.

Column chromatography or HPLC can also be used to purify library components (34,35). Commercial HPLC systems with robotic liquid handling capabilities are available for this purpose. However, until recently this was limited to serial mode. "Plug"-type filtrations, for the removal of polar impurities through silica, can be performed in parallel similar to the SLE process described earlier (14,16). More rigorous methods for parallel purification are starting to appear. A fully automated parallel HPLC-MS system for both purification and characterization of library

compounds has been reported and commercial systems for parallel flash chromatography are now also available (36-38). While a number of approaches have been described, high-throughput purification remains an arduous process.

Library Analysis - The primary tool for library analysis has been mass spectroscopy (MS), by flow-injection analysis (FIA) often coupled with HPLC (39-41). Large libraries present throughput limitations, so a statistical sampling protocol is often applied when a library is analyzed. However a new technique for MS analysis using flow injection methods and a multiple probe autosampler should minimize the need for statistical sampling of libraries that are currently analyzed by MS (42).

HPLC-UV analysis, when linked to MS analysis, is the method of choice. However, throughput constraints are the downside to this approach. The automated parallel system, discussed in context of library purification, is a possible solution to this problem, or at least an excellent compromise. An interesting report recently described the deconvolution of a discreet pooled library using a combination of screening for biological activity and HPLC (43). The libraries were first assayed and the active pools purified by reverse-phase HPLC. The individual fractions were then re-evaluated in the bioassay, as well as by HPLC and LC-MS, to determine structures of the active components. In most cases overlap between fractions was less than 10% for a specific compound.

Nuclear magnetic resonance (NMR) has recently been shown to be applicable to library analysis, in particular when coupled to a liquid handling system, with or without an HPLC column or HPLC pump (44). Background signals from solvents have been a bottleneck to the development of this technique, but can be circumvented by solvent suppression programs, use of deuterated solvents as the mobile phase, and more advanced pulse sequences during acquisition. Sample analysis can run about three minutes per sample, which can be most useful for smaller libraries. The quantity of analyte determines the quality of the spectrum; often field strengths in excess of 500 MHz are required for acceptable signal to noise ratios.

EXAMPLES OF LEAD IDENTIFICATION AND OPTIMIZATION

This section provides selected examples of combinatorial approaches for the identification of biologically active compounds. The range of diverse structures that have been identified employing combinatorial techniques illustrates the increasing generality of the approach. Although there are now excellent and numerous examples of the use of combinatorial techniques in medicinal chemistry, not all chemotypes are amenable to combinatorial synthesis. The current art is to discern if, when, and how to use combinatorial or parallel approaches (45). This chapter is limited to a discussion of non-peptide libraries that have led to inhibitors of pharmaceutically relevant targets. An excellent comprehensive review of both screening and directed libraries leading to biologically active agents has recently been published (46). In addition, a monograph covering synthetic methodology in combinatorial synthesis recently appeared (47).

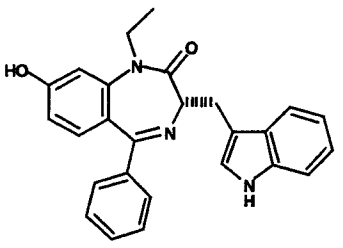
LIBRARIES FOR LEAD GENERATION

The following examples illustrate how leads have been generated from combinatorial libraries designed from known pharmacophores, as well as novel structures that were identified from screening diverse libraries. Reports of libraries that employ similar synthetic methods relative to the specific examples presented here will

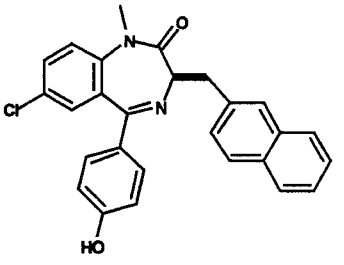
be mentioned in passing, in order to highlight the general utility of a reliable combinatorial process.

KNOWN PHARMACOPHORES

Cholecystokinin Receptor Antagonists - Due to its significance as a motif in many drug classes, the 1,4-benzodiazepine scaffold was chosen early on in the evolution of combinatorial libraries of small molecules. In 1992, the synthesis of these compounds on solid-support was described (48) and later the screening results were reported for benzodiazepine libraries in a cholecystokinin (CCK-A) receptor assay for which the scaffold was shown to bind (49). The structural information combined with a robust assay (using CCK obtained directly from pancreas of a sacrificed rat) made this an appropriate system to demonstrate the utility of screening small molecule libraries. From a relatively small focused library, an 80 nM inhibitor (**1**) was identified (49). Later, from a similar but slightly larger 1,4-benzodiazepine library, several completely novel and selective antagonists, including **2**, were identified against antibodies involved in systemic lupus erythmatoma (50). A library of 40 benzodiazepines related to Valium afforded screening results consistent with the known SAR for these heterocycles (51). The benzodiazepines were prepared with a cyclic cleavage from support as the key step and screened directly for fluoronitrazepam inhibition.

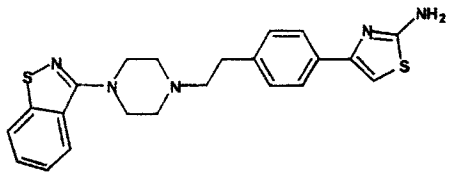


1: IC_{50} = 80.0 nM (CCK-A)



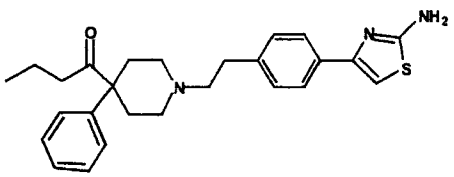
2: 60% inhibition at 20 μM (11F8 mAb binding)

Identification of Herpes Simplex Virus (HSV) Inhibitors - Both solution and solid-phase parallel syntheses has been used as methodology to accelerate the drug discovery process. An early example of targeted parallel synthesis leading to the optimization of both herpes simplex virus inhibitors and neurokinin-2 (NK-2) receptor antagonists was reported from a series of solution libraries (52). Of synthetic interest was the use of the same palladium catalyst for one-pot tandem Suzuki coupling and nitro-reduction reactions. Microwave irradiation to accelerate an alkylation reaction was another novel transformation performed in parallel. Three related libraries were used for the optimization of an in-house aminothiazole derivative with activity against herpes simplex virus. Activity at the micromolar level was readily measured even on the crude library compounds. Exact IC_{50} values for the most active compounds in the library were confirmed by resynthesis and evaluation of purified and fully characterized compounds. These efforts identified **4**, which was 18 times more potent than the lead compound (**3**) in the HSV-1 plaque reduction assay.



3: in-house screening lead

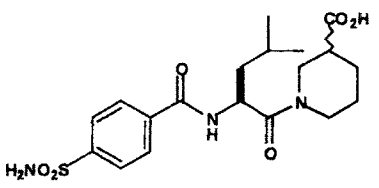
IC_{50} = 3 μM (HSV-1 helicase ATPase)
IC_{50} = 11 μM (HSV-1 plaque reduction assay)



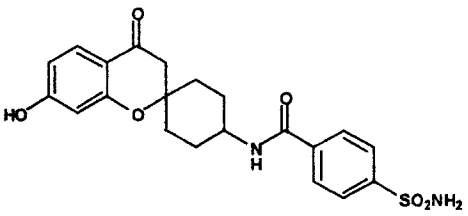
4: optimized lead

IC_{50} = 1.4 μM (HSV-1 helicase ATPase)
IC_{50} = 0.6 μM (HSV-1 plaque reduction assay)

Carbonic Anhydrase II Inhibitors - Combinatorial libraries targeting carbonic anhydrase, have been developed containing the known sulfamoyl (SO_2NH_2) pharmacophore (53). Inhibition of this enzyme is beneficial in lowering the intraocular pressure associated with glaucoma. These libraries were prepared on solid-support, employing tag-encoding techniques. Nanomolar inhibitors **5** and **6**, based on different scaffolds but containing the same 4-sulfamoylbenzamide fragment, were identified against carbonic anhydrase II.

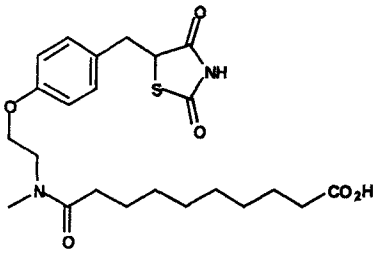


5: K_D = 4.0 nM (carbonic anhydrase II)

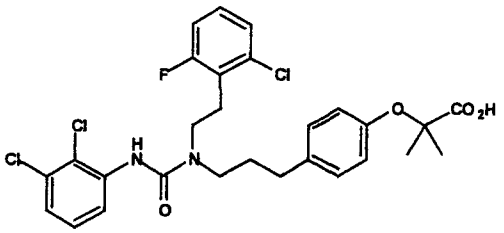


6: K_D = 15.0 nM (carbonic anhydrase II)

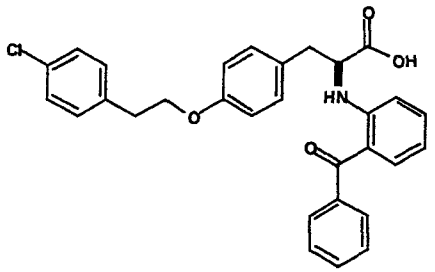
Peroxisome Proliferator-Activated Receptor (PPAR) Ligands - Peroxisome Proliferator-Activated Receptor (PPAR) ligands with antidiabetic activity have been reported using targeted solid-phase synthesis (54). Preliminary NMR analysis suggested that both natural (polyunsaturated fatty acids, FA) and synthetic (thiazolidinedione, TZD) inhibitors adopt turn structures in solution. The hybrid molecules allowed for the exploration of the relationship between natural and synthetic PPAR ligands. Hybrids of thiazolidinedione and fatty acids (TZD-FA) were found to be high affinity human $PPAR_\gamma$ ligands. A 480-member trisubstituted urea library, also targeting $PPAR_\delta$, was prepared, incorporating a phenoxyisobutryic acid monomer commonly found in the birate class of lipid-lowering drugs (55). This biased library showed activity on all three PPAR subtypes and allowed identification of the first high-affinity $PPAR_\delta$ ligand (**8**). Radioligands can now be used in competition-binding assays for new PPAR ligands with improved subtype selectivities. Lastly, a series of $PPAR_\gamma$ ligands was optimized using parallel synthesis of ether derivatives *via* the Mitsunobu reaction of a support-bound N-(2-benzoylphenyl)tyrosine with a variety of alcohols (56).



7: K_i = 18 nM (PPARγ)

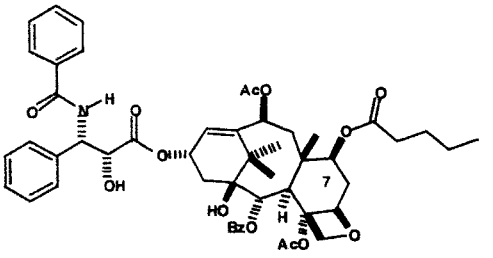


8: IC_{50} = 160nM (PPAR$_\delta$)



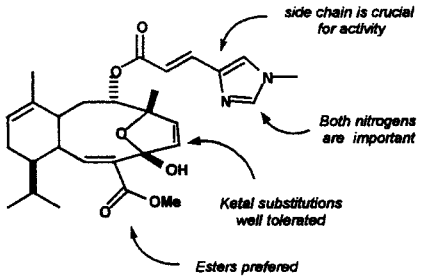
9: pK_i = 8.03 (PPARγ)

Bioactive Natural Products as Lead Structures - Natural products have been a well-known source of medicinally important substances. Recently, three classes of natural products, each possessing antitumor activity formed the basis of combinatorial libraries. Libraries based on the approved chemotherapeutic agent Taxol™ have been the focus of several publications with the objective of identifying analogues with an improved profile for formulation and multiple drug resistance in cancer therapy. A 26-member library of esters at the C-7 hydroxyl of taxol identified four analogues, including **10**, which were more potent in the MCF7-R cell cytotoxicity assay (57). A library based on a series of marine natural products, the sarcodictyins, isolated from a Mediterranean coral, was reported, using combined solution/solid-phase synthetic methodologies (58). The natural products were also synthesized according to the chemistry developed for library generation. A small library of sarcodictyin derivatives was prepared and evaluated for antitumor activity through tubulin polymerization and microtubule stabilization, resulting in elucidation of the sarcodictyin SAR and identification of 10 analogues of equal, or more potent biological activity than the natural products. Libraries based on the epothilones A and B, natural products isolated from the myxobacterium *Sorangium cellulosum* strain 90, have also been described. The library identified nine analogues with IC_{50} values below 10 nM in a cytotoxicity assay against breast and ovarian cancer cell lines (59).

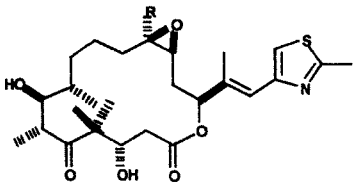


10

$ED_{50}/ED_{50(taxol)}$ = 0.70 (microtube assembly)
$ED_{50}/ED_{50(taxol)}$ = 0.40 (MCF7-R cell assay)



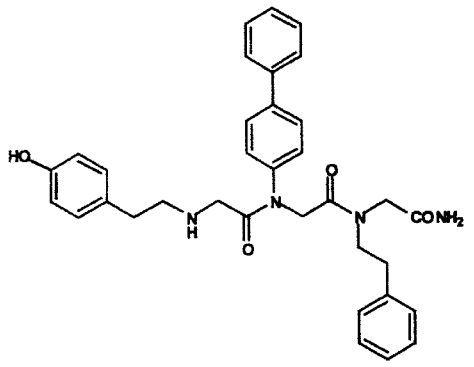
SAR for sarcodictyin A based on library screening data



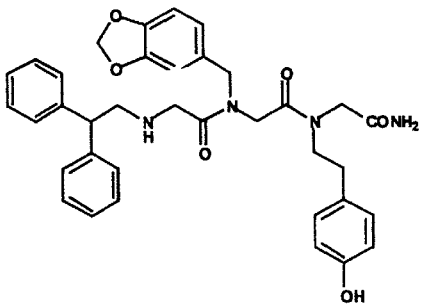
11: R = H; epothilone A
12: R = Me: epothilone B

NOVEL SCAFFOLDS AND STRUCTURES

7TM/GPCR Ligands - In one of the early examples of a non-peptide library, high-affinity ligands to 7-transmembrane G-protein-coupled receptors (7TM/GPCR) were identified from the split synthesis of a diverse peptoid (N-substituted glycinamides) library (60). The most potent structures, **13** and **14**, were structurally distinct from previously reported 7TM/GPCR ligands. A number of other active peptoids have also been reported against a variety of targets, including inhibitors of the signaling protein ZAP-70, a possible target for immunosuppressive agents (61).



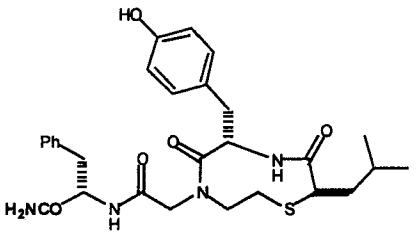
13: IC_{50} = 5 nM (α-adrenergic receptor)



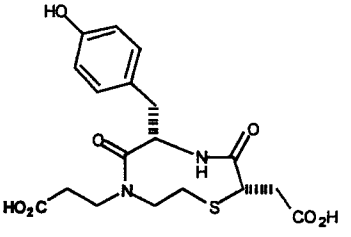
14: IC_{50} = 6 nM (μ-opiate receptor)

As an attempt to move away from linear oligomers containing amide bonds, many reports have appeared that combine the reliable methodology employed for the synthesis of peptoids with more complex heterocycle synthesis, including tetrahydroisoquinolines (62), 1,4-benzodiazepine-2,5-diones (63), and diketopiperazines (64).

β-Turn Mimetic Libraries - The β-turn is a crucial secondary structural motif in peptide and protein interactions, and is involved in many biological recognition events, including binding of peptide hormones and their receptors. Libraries of β-turn mimetics therefore can serve as a source for identifying new structures for inhibitors for a variety of protein-protein interactions. A β-turn mimetic library targeting the fMLF receptor, which is located on the plasma membrane of neutrophils, has been reported (65). Antagonists of this receptor have promise as antiinflammatory agents and potential drugs for infectious diseases. Disclosure of a related library of 2302 β-turn mimetic as inhibitors of binding to the $\alpha_4\beta_1$ integrin site, found along the connecting segment 1 (CS1) of fibronectin, which may lead to potential inflammatory agents (66). The chemistry to prepare these libraries utilized a novel disulfide linker which, when reduced, afforded a sulfur nucleophile that cyclized onto a α-bromoamide at the i+1 position of the turn. In addition, a solid-supported guanidine base was employed to scavenge the reducing agent from the reaction mixture.



15: IC_{50} = 10.0 μM (fMLF)



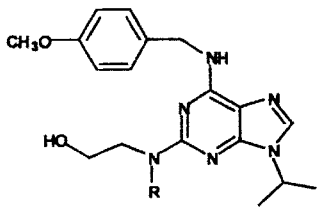
16: IC_{50} = 5 μM (α4β1 binding)

LIBRARIES FOR LEAD OPTIMIZATION

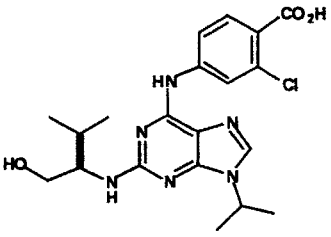
The tasks of lead identification and lead optimization are inherently different problems. Both can use combinatorial synthesis, however the chemistry and precursor selection will be quite dependent on the final targets. Often for lead optimization similar building blocks will be included in the library whereas for lead generation a more diverse set will be required. Examples of libraries for lead optimization with leads are discussed below with different leads ranging from random structures to those prepared based on an understanding of the mechanism of action with specific enzymes.

Cyclin-Dependent Kinases - The cyclin-dependent kinases (CDK's) are enzymes involved in regulation of cell growth, DNA replication and cell division. Inhibitors of these enzymes may have benefits for the treatment of cancer and restenosis. The combinatorial synthesis for the optimization of olomoucine, a selective inhibitor of CDK2 has recently been described (67). From a relatively small library of 348 2-

(acylamino)-6-aminopurines, an inhibitor (**16**) with an IC_{50} of 600 nM was identified. A related, selective inhibitor of CDK2 (CVT-313), was also identified from a combinatorial library (68). Using the injured rat carotid artery model, CVT-313 (**17**) was shown to inhibit restenosis at a dose of 1.25 mg/kg (69). The related purine derivative purvalanol B (**18**), a nanomolar CDK2 inhibitor has been disclosed, along with the structural requirements for its binding affinity from X-ray data on a complex with the protein and inhibitor (70).



16: R = H: IC_{50} = 600 nM (CDK2)
17: R = 2-hydroxethyl: IC_{50} = 500 nM (CDK2)

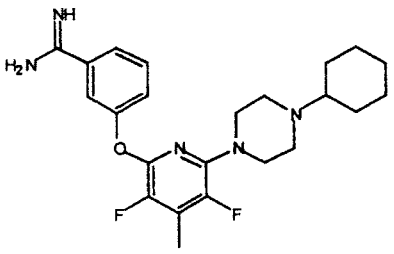


18: IC_{50} = 6 nM (CDK2)

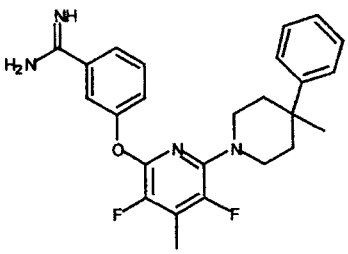
PROTEASE TARGETED LIBRARIES

Combinatorial libraries targeting protease inhibition have recently been a major focus for lead identification and optimization. Specifically, a number of libraries based on known motifs for serine protease inhibition have appeared. In addition, combinatorial and parallel synthesis efforts that target cysteine and aspartyl proteases have also been undertaken. Examples of these libraries are given below, with emphasis on those directed toward thrombin and factor Xa, two proteases involved in blood coagulation.

Factor Xa (FXa) Inhibitors - Factor Xa (FXa) inhibitors (**19** and **20**) have been prepared using solid-phase synthesis by linking the molecule to support through an aryl amidine (71). The aryl amidine moiety was selected because of its known activity against trypsin-like serine protease inhibitors including FXa. This strategy allowed all the products to be synthesized with a pre-formed amidine, avoiding the harsh conditions (e.g. Pinner amidine synthesis) on solid support often required to convert a nitrile to this functional group.

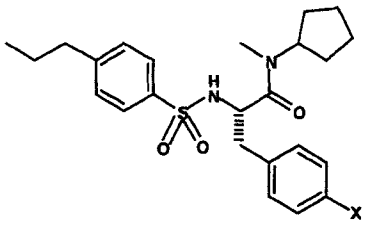


19: K_i = 495 nM (FXa)



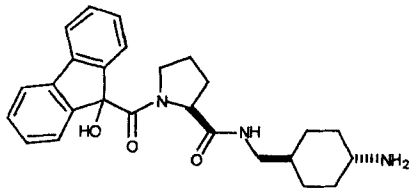
20: K_i = 560 nM (FXa)

Thrombin Inhibition – A library of sulfonamides targeting thrombin and employing an approach similar to that of the FXa inhibitors has been described (72). The library compounds were obtained in good yields with K_i's ranging from 0.1 to 0.01 μM against thrombin. A related library employing a resin-bound 4-(aminomethyl)phenylalanine derivative afforded a thrombin inhibitor equipotent (**22**) and identical in backbone structure to the benzamidine derivative (**21**) cited above (73). As with the factor Xa inhibitors, all of the compounds were linked to the solid support *via* the basic functionality.

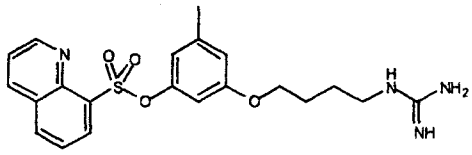


21: X = $C(NH_2)$=NH, K_i = 10 nM (thrombin)
22: X = CH_2NH_2, K_i = 10 nM (thrombin)

Several reports of methodology to generate thrombin inhibitors *via* parallel synthesis have been described. Two examples afforded analogues based on the known thrombin inhibitor sequence D-Phe-Pro-Arg (74). In one case, the P_2-P_1 fragment was attached to an acid cleavable carbonate resin, then isosteres of the P_3 residue were assembled using standard amide bond-forming conditions. This approach allowed for the preparation of some 200 analogues where **23** was identified as a potent (K_i = 1.5 nM) inhibitor of thrombin, exhibiting oral bioavailability in both the dog and rat. The other example applied a nucleophile-cleavable linker (Kaiser oxime resin) to rapidly generate some 20 amides at P_1 from a common, support-bound P_2-P_3 fragment, also based on the same tripeptide sequence (75). Lastly, solution phase strategies with scavenger resins were employed to prepare in parallel a series of arylsulfonyl esters containing a guanidinobutyl moiety as the P_1 element (76). A potent and selective thrombin inhibitor (**24**) was identified from some 35 compounds screened.



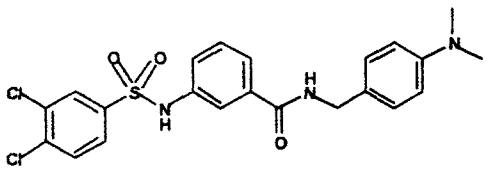
23: K_i = 1.5 nM (thrombin)



24: K_i = 0.0220 μM (thrombin)

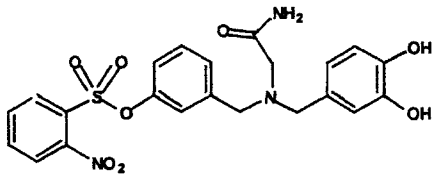
Serine Protease Probe Libraries - A series of probe libraries directed towards serine proteases have been reported, also based on an aryl scaffold (77). High throughput screening of these libraries identified a number of low micromolar inhibitors against different serine proteases. Two examples (**25** and **26**) are shown below. A report of libraries of constrained β-strand mimetics to produce protease superfamily inhibitors

(78) have also appeared. These libraries identified a picomolar thrombin inhibitor (**27**), in addition to nanomolar inhibitors of kallikrein, tryptase and trypsin. The core 1,2,4-triazoline-3,5-dione bicycle was generated on support using a Diels-Alder reaction of a urazene with a support-bound diene; a related approach to prepare screening libraries containing this heterocycle was also recently reported (79).



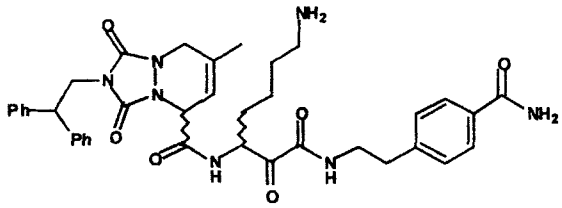
25

K_i = 1.9 μM (Thrombin)
K_i = 0.6 μM (Plasmin)



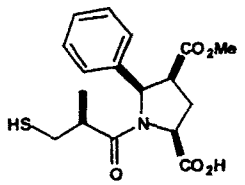
26

K_i = 4.5 μM (Thrombin)
K_i = 1.8 μM (Elastase)
K_i = 0.8 μM (Plasmin)



27: K_i = 0.035 nM (thrombin)

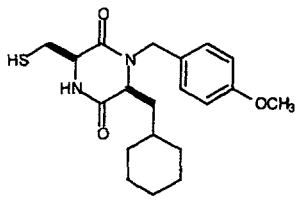
Angiotensin-converting Enzyme (ACE) Inhibitors - ACE inhibitors, typified by captopril and related molecules targeting hypertension and congestive heart failure, are one of the most important drugs classes on the market today. A series of libraries based on the known ACE inhibitor captopril have been described (80). Both spatially separate and encoded libraries of pyrrolidines were prepared employing Grigg's 1,3-dipolar cycloaddition to provide analogs more potent than captopril (81). The different diastereomers were purified to identify the most active component (**28**) from the various mercaptoacyl proline libraries. This mercaptoacylproline was approximately 3-fold more potent than captopril in the inhibition of rabbit kidney ACE. Other reports have appeared employing the mild reaction conditions of the 1,3-dipolar cycloadditions with azomethine ylides for library generation (82,83).



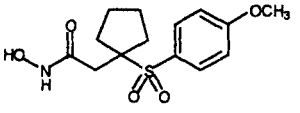
28: K_i = 0.16 nM (ACE)

Another library targeted towards the ACE was disclosed, based on enalapril analogues (84). Other potent chemotypes were identified, albeit none more effective than the original structures.

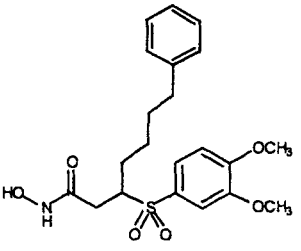
Matrix metalloproteinase (MMP) Inhibitors -The matrix metalloproteinases (MMP's) are an important class of zinc-dependent proteases involved in degradation and remodeling of the extracellular matrix. Inhibitors of these enzymes have therapeutic potential in cancer, arthritis, osteoporosis, and Alzheimer's disease. Diketopiperazine (DKP) libraries designed to target MMP's have been designed, prepared and evaluated for their ability to inhibit collagenase-1, stromelysin-1, and gelatinase-B substrate hydrolysis (85). The DKP libraries were designed to employ the thiomethyl functionality of cysteine to bind to the enzyme's bound zinc, and the remaining two diversity sites to interact with the enzyme's specificity pockets. Deconvolution of active pools resulted in the identification of potent inhibitors of collagenase-1 and gelatinase-B, with the most potent inhibitor (**29**) exhibiting an IC_{50} of 30 nM against collagenase-1. Nanomolar inhibitors of both MMP (**30**) and PDE4 (**31**) targets from similar arylsulfonyl hydroxamic acid templates have been reported (86). Both classes of compounds are available by either solution- or solid-phase routes from the same set of chemical transformations.



29: IC_{50} = 30 nM (collagenase-1)



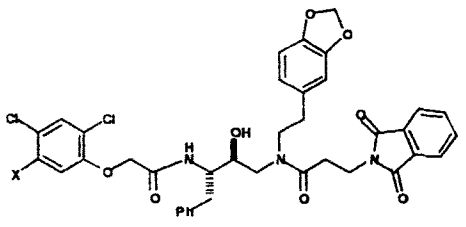
30: K_i = 0.01 μM (MMP-2)



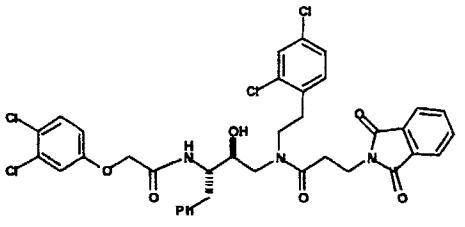
31: IC_{50} = 0.001 μM (PDE4)

Mimics of the tetrahedral intermediate of amide hydrolysis - A series of papers have appeared describing the synthesis on solid support of (hydroxyethyl)amine isosteres displaying non-peptide functionality. The (hydroxyethyl)amine isostere targets aspartyl proteases by mimicking the tetrahedral intermediate in amide hydrolysis. In their initial report, a number of known orally available HIV protease inhibitors were synthesized to demonstrate the generality of the solid-phase methodology (87).

Cathepsin D Inhibitors - This parallel solid-phase synthetic methodology was combined with structure-based design to identify low nanomolar inhibitors of cathepsin D (88). Both diverse and directed side chains were first examined computationally using a docking algorithm based on the known X-ray crystal structure of cathepsin D, and then experimentally based on the assay results from screening different libraries for cathepsin D inhibition. Potent nonpeptide inhibitors had not been previously reported. After screening the libraries, inhibition constants were determined for several of the 'hits' from the designed and diverse libraries. Optimization of the most active inhibitor, **32**, (x = Cl, 73 nM) from an initial library afforded a very potent series of compounds (**33** and **34**), containing different phenethyl amines.

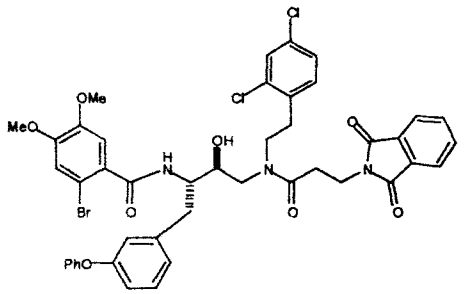


32: x = Cl, K_i = 73 nM (Cathepsin D)
33: x = H, K_i = 9 nM (Cathepsin D)

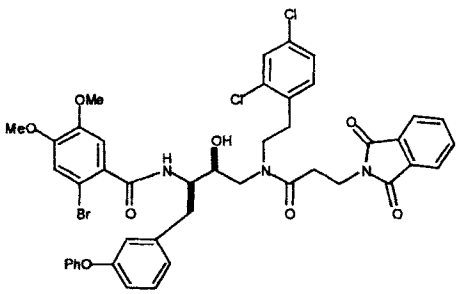


34: K_i = 14 nM (Cathepsin D)

The methodology to prepare additional diastereomers could be expanded by utilizing both enantiomers of isopropylene glycerate attached to solid support. The other chiral centers required for all four possible diastereomers were introduced *via* stereospecific reduction of ketones derived from the glycerate esters (89). This route ultimately resulted in a 700 pM inhibitor of cathepsin D (**35**), where the minor diastereomer (**36**) was at least 7000 times less active. Development of this route also allowed for the preparation the (S)-hydroxyl isomer, essential for improvement in potency over the original set of library compounds.

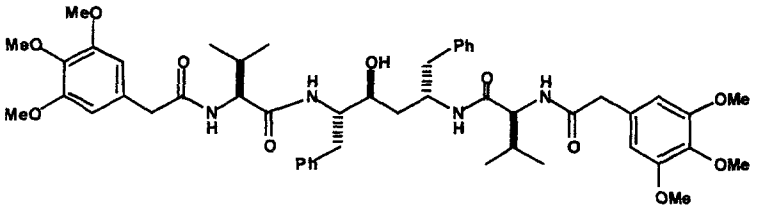


35: K_i = 0.7 nM (Cathepsin D)

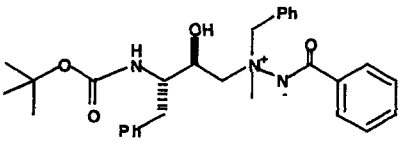


36: K_i > 5000 nM (Cathepsin D)

HIV Protease Inhibitors – Solid phase synthesis of HIV protease inhibitors was undertaken to generate large numbers of analogues and identify potent (IC_{50} <100 nM) inhibitors with enhanced bioavailability and pharmacokinetics relative to the initial leads (90). A library of 300 discrete compounds containing both the diamino diol and diamino alcohol cores were synthesized from resin-bound ketals and acetals derived from these core fragments. Unpurified samples were assayed using concentrations based on the weight and not corrected for the impurities. In general, the mono-hydroxy compounds were more potent than the corresponding diols as exemplified by HIV protease inhibitor **37**. In addition, a new class of HIV-1 protease inhibitors have been reported from a library of aminimide isosteres of the diamino alcohol core found in many aspartyl protease inhibitors (91). Solution-phase reaction of an ester, a 1-methyl-1-substituted hydrazine, and a BOC-amino epoxide afforded the library of aminimide peptide isosteres, from which the most potent inhibitor, AQ148 (**38**; IC_{50} = 0.2 μM), was identified.



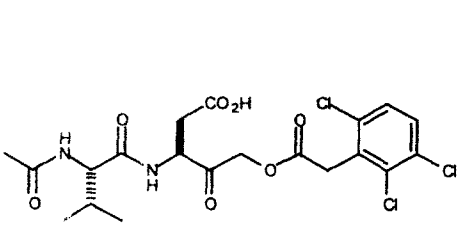
37: IC_{50} < 1 nM (HIV)



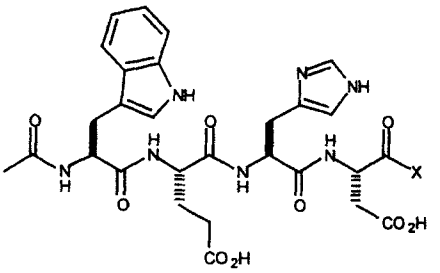
38: IC_{50} = 0.2 μM (HIV-1)

Hydroxyethyl amine libraries have also been prepared in solution from the reductive amination of aminoalcohols with ketones to prepare analogs of known adrenergic antagonists such as pindolol (92).

Cysteine Protease Inhibitors - Parallel methods to rapidly optimize reaction conditions to ultimately generate a series of interleukin-1β converting enzyme (ICE) inhibitors has been described (93). These efforts resulted in the synthesis of 590 compounds pure enough for screening. The best of these, **39**, possessed an IC_{50} of 0.007 μM, after resynthesis and purification. A positional scanning library approach afforded a series of ICE directed libraries derived from peptide-AMC derivatives of aspartic acid as P_1 residue (94). This library led to a peptide sequence Typ-Glu-His-Asp, which was not known for specificity to ICE, and led to the identification of the highly potent aldehyde derivative (**41**; K_i = 56 pM). This example illustrates the powerful advantage of combinatorial chemistry to rapidly identify structural motifs that may not be known for a particular biological target. These positional scanning libraries will be extremely valuable in future medicinal chemistry research for determining new chemotypes that bind to targets, where little or no information on structural requirements for inhibition or binding may be known.



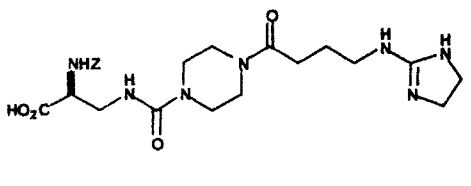
39: IC_{50} = 0.007 μM (ICE)



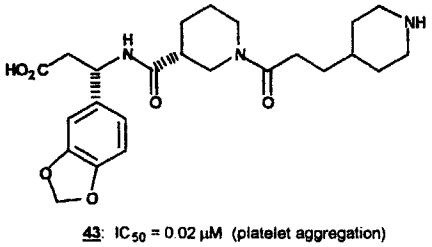
40: X = AMC; hit from substrate probe library
41: X = H; K_i = 56 pM (ICE)

LIBRARIES DIRECTED AGAINST RECEPTOR TARGETS

Fibrinogen Receptor Antagonists - The search for an antagonist of the fibrinogen receptor as a potential antithrombotic agent has been a vigorous area of research over the last five years. A review covering combinatorial approaches to these adhesion receptor antagonists has been recently published (95). A selective antagonist with increased activity was observed from a focused RGD peptide mimetic library (96). The most active compound possessed a urea linkage to a piperazine (**42**). This RGD peptide mimetic was a potent $\alpha_v\beta_3$ integrin antagonist that showed excellent specificity relative to $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_3$, despite the closely related nature of the integrin targets. Solid-phase parallel synthesis was employed to optimize a prototype GPIIb/IIIa antagonist. After synthesis of 250 targets and some asymmetric synthesis of specific hits, β-aminoacid derivative **43** was identified as a potent *in vitro* and *in vivo* mediator of platelet aggregation (97).

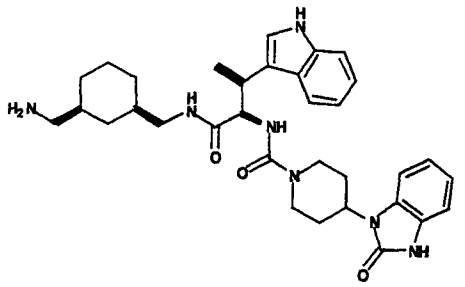


42: IC_{50} = 1.1 nM ($\alpha_v\beta_3$ Elisa)

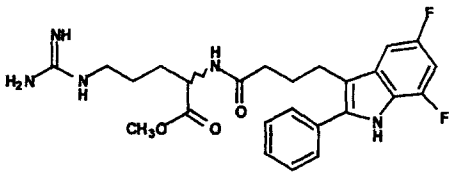


43: IC_{50} = 0.02 μM (platelet aggregation)

Somatostatin Agonists – Libraries targeting the somatostatin receptor have been recently reported (98). Based on a lead structure identified from molecular modeling and a database search, a series of libraries were designed and prepared, resulting in potent and subtype-selective receptor agonists. Selective agonists for all five subtypes of the somatostatin receptor were identified; two examples include L-779,976 (**44**) and L-803,087 (**45**). Since somatostatin inhibits the secretion of growth hormone and insulin, agonists of the somatostatin receptor have therapeutic potential in the treatment of growth hormone disorders and diabetes.



44: IC_{50} = 0.05 nM (cAMP accumulation)



45: IC_{50} = 0.2 nM (cAMP accumulation)

CONCLUSIONS

The examples presented in this review highlight some of the more interesting structures identified by combinatorial chemistry and parallel synthesis. As mentioned earlier, not all chemotypes can be expanded into a library due to the limitations in the synthetic methodology required to generate the target structures. The expansion of the synthetic toolbox of both solid- and solution-phase reactions, as well as the development of more versatile synthesis workstations, will enable chemists in the future to build libraries from a greater number of chemotypes. The difficult part of drug discovery will not be the identification or optimization of an active lead, but rather the conversion of an active lead into a marketable drug.

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Chapter 28. Recent Developments in Molecular Diversity: Computational Approaches to Combinatorial Chemistry

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Introduction – The first "designed" combinatorial library (1) combined elements of medicinal chemistry bias and diversity selections. The underlying mechanics and approaches used for this library design were encoded into a more formal computational approach to the analysis, design, and synthesis of combinatorial libraries (2). This new field of molecular diversity has since expanded to match the rapid growth in high-throughput screening (HTS) and synthetic methods for the production of combinatorial libraries. In the early days of combinatorial chemistry, molecular diversity was seen as a different field distinct from chemical information and molecular modeling. This distinction is no longer valid -- chemical informatics, molecular modeling, and molecular diversity are inseparable.

This review will attempt to summarize the advances in the field of molecular diversity defined as the computational analysis of large numbers of chemical structures and related biological data. Several recent reviews have appeared elsewhere that delve more deeply into specific areas of computational analysis (3-11).

The primary aim of molecular diversity methods is to reduce the excessively large number of potential starting materials to a manageable synthetic plan. The approaches employed today can be broadly classified into three areas. Most diversity methods rely on the principle that active molecules share high "similarity" to other actives. In the first approach, one is interested in selecting a more or less different (dissimilar or diverse) subset of the molecules within the starting pool of compounds, so that the chance of finding a hit in screening is maximized. This method is used for general or diverse libraries for broad screening and for plugging holes in a corporate library. For lead optimization efforts, a second approach is employed, in which one selects similar molecules. Finally, an emerging area of interest relates to methods that allow for the jumping of one chemical class to another to provide, for example, novel compound classes as backups for clinical candidates (12-15). These latter methods are based on a three-dimensional description of the chemical similarity.

The advent of combinatorial chemistry methods has significantly changed how compounds are evaluated and designed. Computational chemists no longer focus on just one or two compound selections; they now must suggest hundreds to thousands of compounds. This has led to the invention and testing of many new computational methods, which has led to an explosion of new methods.

This explosion of new approaches has led to a plethora of acronyms, algorithms, and competitive methods. In addition, these methods require a substantial amount of manipulation both on input and on output. Ideally, these methods would be easy-to-use and available to most, if not all, medicinal chemists. Unfortunately, it is unlikely this will happen in the near-term and it is likely that experts will continue to be required to carry out library designs. Nevertheless, it does appear that molecular diversity approaches are converging, rather than diverging. One can generally categorize molecular diversity methods into 1) filtering methods which allow for the elimination of unwanted or undesirable chemical functionality, 2) diversity metrics which describe how two compounds relate to each

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other and 3) selection methods to reduce the number of possible choices to a manageable number, and 4) "virtual" library methods – compound sets that exist computationally, rather than physically. Structure-based design methods fall under the scope of this last category.

Computational Filters -- Diversity methods usually have as common starting point a pool of possible or accessible compounds either as individual molecules or reactants. Regardless of the method employed, the list of potential molecules far exceeds synthetic capabilities (and perhaps sensibilities) and filters must be used to narrow the list to a manageable number. Filtering of lists requires a combination of computational filters, medicinal chemistry intuition, and synthetic considerations (2, 11, 16). List filtering is most often described in scant detail, perhaps because it is obviously needed, relatively simple, and not terribly exciting. However, if done improperly, immense amounts of computational resources can be wasted and the desired selection is compromised.

Topological computational filters are used in a general manner to remove reactive, flexible, large, and otherwise unattractive compounds from a source pool. Reactive compound classes and those that are known to provide false positives in high throughput screening are often removed prior to a more expensive calculation. These filters are applied as substructural queries. After this general filtering, a more specific filtering must be employed. Each specific chemistry must be dealt with individually. The most critical aspect of filtering is the interactive process of working with the synthetic medicinal chemist to achieve an acceptable list of compounds for synthesis.

Diversity Metrics and Molecular Filters -- Diversity metrics and molecular descriptors are synonymous and refer to the computational evaluation of the similarity of two compounds. Diversity metrics can be subdivided into those that require the use of sidechains (reagent-based) and those that use whole molecules. They can also be divided into those that use topological descriptors (2D) and those that require conformationally dependent descriptors (3D). This area has been reviewed (3, 5, 7).

Most of the molecular diversity approaches have relied on some form of 2D property calculation. In the original work, Martin, et al., used a reagent-based approach combined with 2D properties and calculated logP using linear regression and multi-dimensional scaling methods for their molecular descriptors (2). This has been supplemented with a method to catalogue sidechains in pseudo-3D using a molecular docking technique and with pharmacophores calculated from the sidechain (16, 17).

More extensive three-dimensional approaches have also been employed. The most common metric extends pharmacophoric 3D database queries to combinatorial chemistry approaches. These 3D queries have been used successfully in the identification of several novel classes of inhibitors (9, 14, 18-20). In these and related approaches, a set of molecular features of interest are defined (e.g., H-bond donors and acceptors, positive and negative charges, and aromatic rings and hydrophobic groups) and are mapped onto the conformations of a molecule. If the distances are binned into distance ranges, then the entire set of 2, 3, or 4-point pharmacophores can be calculated and mapped into large bit string (also called molecular fingerprints or signatures). The pharmacophores that are exhibited by any given molecule can then be mapped into this bit string. Similarity of two molecules is calculated as in the 2D metrics.

Unfortunately, there are some drawbacks to the pharmacophore approach. For example, there is no steric information included in the pharmacophores. This can

result in cases in which two molecules which share a common pharmacophore are in effect superimposed in an unrealistic manner. In another extreme, each simple pharmacophore might be shared with thousands of molecules. A database search with such a ubiquitous pharmacophore often yields many false positives. One might be tempted to add additional features to the simple pharmacophore. In doing so, however, the number of potential combinations of pharmacophoric features and inter-feature distances increases exponentially. These multi-feature pharmacophores (e.g. those obtained using the program Catalyst are more complex and much slower to calculate (21). On the other hand, one can quickly build pharmacophores that are extremely specific for a given class of molecules. A database search with such a discriminating pharmacophore would return nothing, precluding any possibility for lead evolution.

These shortcomings have been addressed by using ensembles (or sets) of pharmacophores selecting those that return fewer hits against a set of random compounds (20). Additional constraints such as steric exclusions can also be added.

Several methods have been developed to include shape in diversity metrics. In the Compass method, the H-bond acceptor, H-bond donor, and hydrophobic characteristics of a molecule are projected onto the molecular shape (21). This 3D surface-feature method is used in the IcePick approach which defines the similarity of molecules based on their surface features (22). Full conformational flexibility is included by a random search and minimization. The similarity of two molecules is evaluated by docking one molecule into the shape of the second molecule. This provides a fixed mapping of two conformations of the molecules. The dissimilarity is defined as the difference in the ability of the two molecules to exhibit the same features on the common shapes of the molecules.

An extension to existing three-dimensional QSAR methods have been used to allow for the rapid evaluation of the bio-isosterism of a set of reagents (23). In this approach, the steric molecular field is computed for all substituents of a core chemical class. Ostensibly, the "core" is aligned, and a single conformation of the substituent is used. Substituents are evaluated as similar if they produce steric fields that are similar.

Semi-empirical methods have been utilized for diversity metrics. The BCUT parameters (a conjunction of three names: Burton, Chemical Abstract Service, and Univ. Texas) are an interesting and somewhat unusual metric (24). These six-dimensional descriptors are calculated rapidly using semi-empirical methods based on a 3D model of the compound of interest. Three small matrices are constructed and then diagonalized. The highest and lowest eigenvalues are combined to form a six-dimensional descriptor.

The use of both 4-point pharmacophores and a molecular shape combined with a molecular feature (shape/feature) for the analysis of compound libraries has been described (15). Both methods can be used to create molecular fingerprints of compound libraries, and rely on the 3D properties of the molecules. Both methods can be used to search large virtual libraries. Since these are 3D methods, it is a requirement to have a fast and complete conformational analysis method. The conformational analysis method used in combination with these metrics has recently been described (25).

There is no universally acceptable metric of "chemical or molecular diversity". In fact, there seems to be almost a religious fervor attached with diversity metrics and sidechain vs. whole-molecule approaches. This results in a field with many different methods, each of which is highlighted with the method's strengths. Comparison of one method to another invariably results in one method theoretically

"outperforming" another. As an example, some reports have appeared that conclusively demonstrate that reagent-based approaches are inferior to whole-molecule approaches (26). However, just as strong an argument is made for the reagent-based approaches (16).

Selection Algorithms -- Selection is the next stage in the process, whether for design of combinatorial libraries or for plugging holes in a corporate compound collection. A wide variety of selection methods is available and has been reviewed (11, 27, 28). Methods can be seen as falling into one of three classes: 1) coverage methods, also known as partition-based or binning methods, 2) diversity methods, characterized by the emphasis of the similarity or dissimilarity of compounds, and 3) informative methods.

In coverage methods, one catalogues compounds in regions of space and selects a single molecule to represent that region of space. Once a molecule has been selected, this area has been "covered". The ultimate goal is to cover as much space as possible with as few compounds as possible.

One of the most widely used selection methods is clustering, in which source pool molecules are catalogued (or selected) into subsets of nearest neighbors based on the diversity metric chosen. Several reviews of clustering approaches have appeared in the literature and will not be repeated here (8, 29-34). However, clustering techniques fall into one of two general classes, hierarchical and non-hierarchical. The hierarchical approaches can be divided into those that develop clusters by successively dividing databases into smaller and smaller clusters (divisive methods) and those that develop clusters by successively grouping compounds together (agglomerative methods). Both continue until a predetermined number of clusters has been achieved. The non-hierarchical methods, such as the Jarvis-Patrick method, group molecules into clusters based on their common nearest neighbors (35). These methods have the advantage of being applicable to larger datasets.

The effectiveness and compatibility of several 2D and 3D diversity metrics used in combination with several types of clustering methods has been studied (31, 35). The compatibility of a given metric with a given clustering method was evaluated on the combination's ability to cluster active molecules with other active molecules. They show that hierarchical clustering as a selection method combined with MACCS 2D keys as a similarity metric are superior to pairings of other methods. They also show that 3D database keys are poor when combined with any clustering method. The Jarvis-Patrick clustering method was shown to be poorer than other clustering methods when combined with many diversity metrics. This is particularly of note since J-P clustering has been widely used because of its suitability in clustering large databases (>50,000 compounds). Other clustering methods are appropriate for only relatively smaller databases.

A distinct disadvantage of both hierarchical and non-hierarchical clustering methods is that compounds cannot easily be added to the dataset without re-clustering. Recently, a stochastic clustering method has been developed in which a set of dissimilar probe molecules are selected and the remaining compounds within the dataset are grouped around these probe molecules (36). This method is rapid and can accommodate additional compounds without having to re-cluster the entire set.

Similar approaches have been used to select compounds to supplement corporate databases (37, 38). These methods have been described as "filling holes" in a corporate collection. It is not cost-effective to purchase compounds one already owns, so a set of compounds are purchased that are dissimilar to the

current corporate collection. Corporate collections have been clustered and compared with J-P clustering (37).

Other coverage methods have also been used for compound selection (19, 24). In these methods, the volume of chemical space is partitioned into many smaller volumes. Compounds are profiled using a diversity metric and placed into these partitions or bins. For example, in the case of the 3D pharmacophore methods, each bit in the fingerprint represents a small volume of chemical space. Selections proceed so as to select a representative or representatives from each of the smaller partitions (19). Optimization methods are applied to maximize the coverage of all bins. This ensures that the final selection not only spans the extent of chemical space, but also samples the central portions well.

Several methods have been introduced for selecting a "maximally" or "optimally" diverse subsets of compounds (2, 16, 39). These methods can be summarized as mathematical search and optimization methods over similarity space. Compounds are selected so as to span chemical space as well as possible given the source pool. In contrast to the coverage methods, these methods try to minimize the similarity between the compounds selected.

"D-optimal" design algorithms have been employed for selecting a "maximally" diverse set of compounds (2). This method tends to select compounds on the "corners" of the chemical space defined by the diversity descriptor set used. This method requires the creation of a multidimensional space in which compounds are defined as points within that space (16). This can be a very time-consuming step *and is limited* to a small number of compounds (<10,000). Methods that do not require this multidimensional scaling step have an advantage of speed and scalability. Other "optimality" methods have also been used, such as S-optimal method (40).

A stochastic search method which includes a simulated annealing approach has been used to find an optimally diverse subset of large databases (39). This work also describes a method to visualize the results of such a selection – one of the few methods to do so. The use of several other experimental design methods in the selection of molecules within large chemical databases has been described (41). The OptiSim method uses a K-dissimilarity its selection method (42). A selection method called a minimal weighted spanning tree is used in combination with a molecular shape dissimilarity metric (22).

As an alternative to both coverage and diversity selections, methods have been described to select an informative subset of compounds (43). The source pool is profiled using a bit string or binning approach. Instead of applying the coverage or diverse selection algorithms, however, an optimization method is applied to maximize the informativeness of a set of molecules (15, 44). An informative selection is one in which all areas of chemical space is covered with the requirement that all compounds selected must have overlapping sections of chemical space. The overlap is required to be modest, rather than absolute. No overlap would approximate a diverse selection while full overlap would select only compounds that are highly similar. Noise is also considered in the selection algorithm.

A simple question often arises: are these selection methods better than a random selection? A computational analysis has shown that clustering and maximally diverse selections are much better for selecting compounds than any random selection (45). Furthermore, they show that 3D-QSAR methods are enhanced by employing a maximal diversity selection in 2D prior to performing the 3D QSAR. No experimental validation of any one given approach has been suggested.

Early diversity designs tended to be comprised of fairly massive, hydrophobic molecules with many rotatable bonds (46-48). This resulted in combinatorial libraries that produced hits, but were unlikely to be amenable to optimization which would yield lead candidates. To address this shortcoming, many researchers have included penalties in the selection methods or in the pre-filtering stages for avoiding compounds with these undesirable features. The Lipinski "rule-of-5" are an example of these types of filters (49). As another example, Martin, et al., have used these filters to include a profiling of molecular weight, calculated logPs, flexibility, and others (16, 17). As a further refinement, all compounds are binned according to a series of user-specified properties, such as H-bond donor containing, charge containing, fluorine-containing, etc. Final D-optimal selections are accompanied by a coverage-based selection from these bins using parameters that are selected by the medicinal chemists to achieve a more reasonable selection of compounds at the final stage of selection.

Virtual Library Searching and Structure-Based Design -- Virtual library screening has become more prevalent in the past few years (11, 15). In general, this method refers to the computational evaluation of a series of compounds that exist only in a computer representation. The term can be used to define the computational construction of chemical libraries and 2D or 3D database searches within that database. A comprehensive review of virtual library searching has appeared recently (11). This review cites the speed of virtual library screening at about 10,000,000 compounds per month on a multi-processor system. Clearly this is too slow, and they speculate that approximate methods will continue to be used for virtual library screening. A recent implicit virtual library searching method was described that uses these approximations to attain higher throughput (50).

Reviews of the combination of combinatorial chemistry with structure-based design have appeared (51-59). Evidence of the synergy between these methods is seen in the increasing number of papers employing structure-based design as a filter on a combinatorial library design. A few examples include the use of a GA to search a virtual library for thrombin inhibitors using a flexible docking approach (17), to select a combinatorial library using a 3D grid (60), to simultaneously select compounds for synthesis and to optimize their affinity (61), and to design combinatorial libraries with unique molecular weights (62). Combinatorial methods were used with structure-based design in the discovery of Src SH3-domain ligands (63). Molecular docking techniques were combined with combinatorial synthesis to identify low-nanomolar compounds against Cathepsin D (64). The use of pharmacophores generated from the active site of a metallo-proteinase has been used as a method for virtual library screening (65).

An information theoretical approach has been used for virtual library searching. The molecular fingerprints of a set of molecules is calculated (in this case, 3D pharmacophores and molecular shapes with features), and screened against a biological target. The bitstrings are searched to identify those sections that are able to separate actives from inactives. Usually, this is a small fraction of the overall bitstring. Only these sections of the bitstring are considered in searching a virtual library (15). Conformational analysis is performed on the individual components of the virtual library and profiled against the small bitstring. Compounds are selected on their ability to match or not match these bits. This approach has been applied as part of an iterative cycle of lead identification and optimization (15).

Drug-Like Libraries -- Perhaps the most significant recent advance in assessing the design of libraries has been the incorporation of methods to evaluate and bias diversity selections to drug-like properties. Several groups have developed drug-like metrics that can be used as filters for compound source pools. Several

methods have been developed which measure similarity of molecular fragments/moieties in a source pool to a list of known bioactive compounds.

A catalogue of commonly seen molecular shapes of drug molecules has been used to bias libraries (66). These shapes are devoid of atomic information other than bond connectivity and sidechain attachment points.

A measure of "drug-like" in which similarity of potential reagents to selected fragments of the top-selling drugs has been employed (17). A potential reagent is classified as drug-like if it contains at least one of the drug fragments. In an analogous approach, potential reagents are selected based on their similarity to substructural elements from a known drug database (67). An inherent difficulty with this approach is that a reagent is said to be drug-like even if the drug fragment is only a small subset of the biologically active molecule. This has been extended with a method to identify common substructures of biologically active molecules by fragmenting molecules around bonds which are formed by common reactions (68). Potential reagents are compared against smaller, realistic fragments of known drugs, including the chemical environment of the reaction centers. They find approximately 20,000 fragments. These fragments have been made easily accessible internally to the company in an on-line database.

Other methods use an unbiased approach to finding drug-like fragments from large databases of known pharmaceutically active compounds. In one method, a Bayesian neural net is used in combination with MACCS keys to distinguish drug-like from non-drug-like compounds (69). This method is able to correctly catalogue drug and non-drug compounds from known databases about 80% of the time. It is clear from this work that drug-like cannot be determined from topological similarities of compounds. In another method, a neural net and atomic descriptors are combined to develop a scoring scheme for drug-like and non-drug-like compounds (70). Likewise, this method also is able to correctly catalogue about 80% of drug and non-drug databases properly. Both show promise in filtering large databases or source pools in an unbiased manner.

The authors of both studies caution that these filters should not be used as absolute filters for drug-like properties. Since the methods rely on current drugs, it is possible that extrapolations to novel drug-like compounds might be limited. Also, the methods produce a score that is probably best used conservatively to eliminate only those compounds that are clearly below the cutoff threshold of non-drug-like meaning that there is no guarantee that compounds that pass the threshold are drug-like whatsoever.

Others have analyzed the physicochemical properties of databases of biologically active compounds in an effort to derive metrics which will help bias libraries towards compounds with desirable properties. Perhaps the best known of these studies resulted in Lipinski's "Rule-of-5" (49). These "rules" describe a method for evaluating compounds using easy-to-compute methods that help bias libraries away from compounds that might prove to have undesirable physicochemical properties. They are not used as a hard-and-fast rule. Specifically, the rules are used as a metric that poor absorption of permeation are more likely when: a) there are more than 5 H-bond donors, b) the molecular weight is over 500, c) the MlogP is over 4.15 or ClogP is over 5, and d) the sum of Ns and Os is over 10 (49).

The correlation between oral bioavailability and molecular weight, octanol/water partition coefficient, and conformational flexibility has been investigated (71). This group finds that no general rules for enhanced bioavailability can be derived beyond the widely recognized emphasis on low molecular weight and logP between 0 and 3. A more detailed discussion of likely reasons why a good number of orally

bioavailable compounds can fall outside this range is presented. A comprehensive evaluation of the Comprehensive Medicinal Chemistry database has led to "qualifying ranges" for drug-like for each of several computational properties (72). An empirical evaluation of the occurrence of functional groups is also presented. Not surprisingly, benzene is the most common functional group. Distributions of functional groups and calculated properties can be significantly different when comparing compounds having different biological properties. These types of calculations are based on the idea that empirical studies of drugs and non-drugs can provide rapid filters for new compounds. However, more extensive calculations might prove more successful in suggesting why compounds are or are not absorbed.

The polar molecular surface properties of molecules have recently been shown to be correlated with oral absorption (73, 74). The dynamic polar surface area of a drug correlates well with an in vitro model of absorption for a series of nine beta-receptor-blocking agents. A related method has been applied to oligopeptides (75). Extension of the methods beyond the limited datasets will be required before this method is used widely.

Conclusions -- The field of molecular diversity has changed rapidly to meet the assortment of chemical and biological approaches to combinatorial chemistry. Over the past several years, several methods have been introduced, modified, and extended. Disagreement – sometime heated – about the exact computational approach to employ exists. Sometimes there are theoretical arguments that appear to contradict each other. This disagreement should not come as a surprise, given that there are many approaches to combinatorial chemistry and high-throughput screening practiced in the industry today. It is often easy to focus on the differences between methods. However, this review has attempted to provide a basis for understanding and focussing on the commonalties of the molecular diversity methods.

Knowles recently summarized the early days of combinatorial chemistry, "many of us believed that 'numbers' were the answer to diversity. Unfortunately, the diversity we created was often irrelevant, or only semi-relevant to the biology we were interested in interfacing with" (76). Molecular diversity approaches have begun to embrace the biological relevance of combinatorial designs, both in terms of in vitro profiling and now in terms of in vivo profiling. Over the next five years, it is probable that many newer methods will replace some of the methods described herein. Current trends appear to be leading to approaches that are tightly integrated with the synthetic medicinal chemists and are emphasizing the chemical, biological, and pharmaceutical needs, rather than the statistical needs of combinatorial chemistry.

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Chapter 29. Structure-Based Drug Design

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Introduction -- Structure-based drug design is a general term for using protein structural information from x-ray crystallography or NMR spectroscopy to assist in the design of novel therapeutic agents. Structure-based drug design is currently most applicable to developing inhibitors which bind to enzymes. Overall, approximately one-third of marketed drugs function primarily through direct inhibition of enzyme activity (1). Over 200 enzymes have been proposed as therapeutic targets within the pharmaceutical industry (2). Clearly, new enzyme inhibitors have great potential to become therapeutically useful in the clinic and successful in the market.

In this chapter we review only recent examples the design of drugs which inhibit enzymes of therapeutic interest. There have been a number of reviews covering earlier work in this field (3-6). The reader is also referred to reviews on technologies designed to exploit structural information -- docking, Ki prediction, and the design of combinatorial libraries (7-9). Finally, we do not cover recent advances in solving structures of membrane-bound proteins such as integral membrane transporters (10) and ion channels (11). This technology is not yet widely applicable to pharmaceutical applications, but it is rapidly evolving and within the next decade structural biology will begin to play an important role in the design of drugs aimed at these targets.

STRUCTURAL GENOMICS AND STRUCTURE-BASED DRUG DESIGN

Diverse structures and functions of therapeutically relevant targets -- Detailed analysis of drug targets indicates that they cover a very broad spectrum of enzyme activities as well as being structurally diverse (Table 1). This implies that efficient enzyme inhibitor design requires flexibility in the implementation of enzymatic assays as well as an appreciation of the subtleties of protein structure. Data from both structural biology and enzymology must be combined in order to understand enzymatic mechanism at the atomic level. This is essential to fully utilize structure-based drug design.

Fortunately, there is a large collection of diverse protein structures available. There are now more than 9000 structures solved by either x-ray crystallography or NMR spectroscopy in the protein data bank, and the rate of new structure submissions continues to grow (12). This collection of experimental results can be used to construct a much larger database of reliable homology models which indicate not only the global folding pattern of the protein, but also allow inferences to be made of the three-dimensional details of the active site. Of the clearly identifiable enzyme targets (those to which a gene sequence can be associated) that are being pursued, approximately 80% are known to have a structural homolog.

Table 1 - Examples of protein families

Family	Num. related targets	Num. homologs
Protein kinases	40	500-1000
Chymotrypsin-like serine proteinases	16	75-100
Cytochrome p450's	9	50-75
Matrix metalloproteinases	10	15-25
Phosphodiesterases	6	15-25

Recent important structures - Since January 1997 there have been at least 20 new protein structures reported in the scientific literature which have immediate implications for current pharmaceutical research. Some of these are listed in Table 2.

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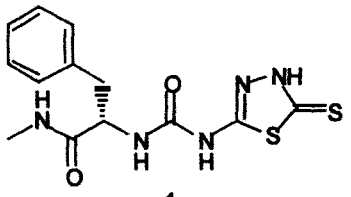
Table 2 – Recent examples of crystal structures of pharmaceutical interest

Structure	Clinical Use	PDB code	Ref.
VEGFR	antiangiogenesis	1VR2	(13)
cPLA2	inflammation	1CJY	(14)
Gyrase A	antibacterial	1AB4	(15)
Tryptase	asthma	1LDT	(16)
Squalene cyclase	hyperlipidaemia	1SQC	(17)
Nitric oxide synthase	inflammation	1NOS	(18)
NF-kappaB	inflammation	1A3Q	(19)
PPAR gamma	diabetes	1PRG	(20)
Cathepsin K	osteoporosis	1ATK	(21)
HCV RNA helicase	antiviral	1A1V	(22)
Topoisomerase I	cancer	1A31	(23)
Insulin receptor kinase	diabetes	1IR3	(24)
JNK3	neurodegeneration	1JNK	(25)
MetAP-2	antiangiogenesis	1BN5	(26)
Histone acetyltransferase	cancer	1BOB	(27)
Cdc25	cancer	1C25	(28)

RECENT EXAMPLES OF STRUCTURE-BASED DRUG DESIGN

Proteases - Proteases would appear to be amenable to structure-based drug design. However, drugs presently exist for only two proteases: HIV protease and angiotensin converting enzyme. The problem of deriving orally bioavailable non-peptides or pseudo-peptides from known peptide remains challenging. This section will focus on recent structure-based design involving proteases, β-lactamases, and neuraminidase.

Matrix metalloproteases (MMPs) - Inhibition of matrix metalloproteases continues to be a very active area of research since the role of MMPs has become more clear in the proteolytic breakdown of basement membranes and angiogenesis. There have been several recent examples of structure-based MMP inhibitor design (29-32) as well as reviews on this topic (6, 33-35). Some noteworthy structures of MMP's incorporated novel zinc-binding groups such as thiadiazole (36). PNU-107859 **1** binds to the S1-S3 portion of the stromelysin binding site as determined by NMR spectroscopy.



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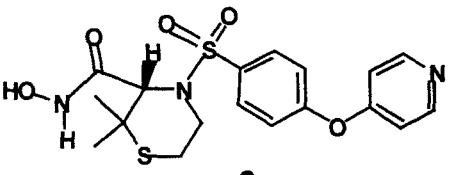

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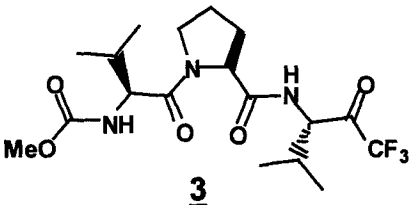
Structural information can play a critical role in understanding the atomic-level interactions of MMP inhibitors. Novel lactam hydroxamates (37) complexed with stromelysin were found to bind in an unexpected fashion. PNU-142769 bound in a flipped orientation with the phthalimide in the P1' pocket. Additionally, some malonic acid-based inhibitors were also found to bind in a nonsubstrate-like binding mode (38).

Design of inhibitors with large P1' groups to complement the stromelysin S1' pocket resulted in AG3340 **2** which has potent inhibitory activity towards stromelysin-1, gelatinase-A, and collagenase-3, but is less potent against collagenase-1 and matrilysin which possess smaller S1' pockets. Utilization of key crystallographic information of a pseudopeptidic inhibitor complexed to stromelysin led ultimately to AG3340. Optimization of the pharmacokinetic profile of these compounds also utilized structural information to suggest which compounds would retain potency (39).

AG3340 is in phase III clinical trials for patients with advanced non-small cell lung cancer, and in patients with advanced hormone refractory prostate cancer.

Serine proteases and related enzymes - The use of structural information in the design of iInhibitors of human leukocyte elastase remains an original and elegant story. β-Lactams based upon structural work done on porcine pancreatic elastase have been reported (40-43). An active site model has been derived of human leukocyte elastase (HLE) bound to a tripeptide trifluoromethyl ketone inhibitor from X-ray structures of HLE and porcine pancreatic elastase. This information was used to aid in the design of a series of novel pyridone-containing, non-peptidic HLE inhibitors (44) which was followed up by further design efforts (45-47) and ultimately resulted in a orally active clinical candidate, ZD8321 **3** (47).



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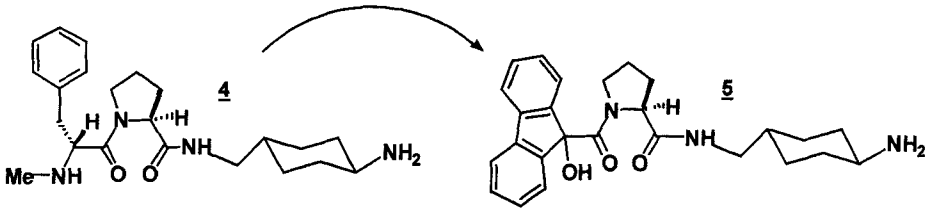
Lessons learned in design of elastase inhibitors have applied to other serine proteases such as thrombin. Both structure-based and classical approaches towards thrombin inhibitor design have been recently reviewed (48-50). The first generation of thrombin inhibitors to reach the clinic were injectable polypeptides. More recent strategies applied to thrombin inhibition has led to novel and selective small molecule inhibitors (51-53), some of which are orally active (54, 55). One case involved the rapid solid phase synthesis of approximately 200 analogues based on a design hypothesis derived from crystallographic data of the lead compound shown below.



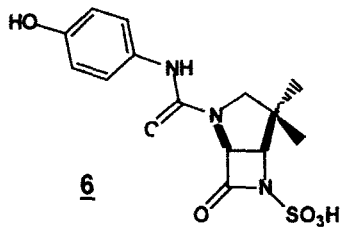
Analysis of the crystal complex of the lead structure **4** suggested that the N-Me-D-Phe at the P3 position could be replaced with larger groups. Compound **4** possessed a K_i = 5.0 nM, but had poor blood levels upon oral administration in rats and dogs. It was reasoned that derivatives of compound **4** might be found which would retain hydrophobic interactions in the S3 pocket while also improving oral absorption and half-life. The solid phase synthesis of approximately 200 diverse lipophilic carboxylic acids chosen from 2200 candidates were coupled to the P3 position of the scaffold which was immobilized through the free amine on a Wang resin. One of the compounds to emerge from this analysis was L-372,460 **5** which had a K_i = 1.5 nM and oral bioavailability of 74 % in dogs and 39 % in monkeys with a serum half-life of approximately 4 h. This compound was efficacious in a rat model of arterial thrombosis and is being evaluated for use in thrombogenic disorders.

Another serine protease target intimately involved in blood coagulation is factor Xa which catalyzes the conversion of prothrombin to thrombin. Recent reviews of this enzyme have appeared (48, 56). The discovery of small molecule inhibitors of factor Xa has been slow relative to thrombin, but structural data has recently appeared (57) and researchers have applied this information and modeling to design cyclic urea (58),

bisbenzamidine isoxazoline (59), and bis-phenylamide carboxylate (60) small molecule factor Xa inhibitors. One interesting aspect of this work is that the modeling was carried out using the non-inhibitor bound structure of factor Xa (61).

Related to the serine proteases are the β-lactamases, responsible for the inactivation of β-lactam antibiotics. They utilize an active site serine hydroxyl as a nucleophile to attack the β-lactam carbonyl and form the acyl enzyme intermediate; the remainder of the β-lactamase catalytic machinery differs from serine proteases. Recent reviews cover general aspects of β-lactamase inhibition (62, 63) and structure-based design (64). One noteworthy example of structure-based design produced novel and selective mechanism-based inhibitors of class C β-lactamases (65). A series of bridged monobactams were designed to stabilize the acyl-enzyme intermediate from further breakdown by blocking the access of water to the enzyme-inhibitor ester bond. Comparison of the bound conformation of aztreonam with the intact conformation of the b-lactam molecule combined with knowledge of the nucleophilic water geometry from the native enzyme structure provided the basis for this design. One inhibitor to arise from this work **6** showed good potency and selectivity for class C β-lactamases from two organisms and synergy with a β-lactamase sensitive antibiotic against Enterobacteriaceae, but not P. aeruginosa.

Cysteine proteases - There have been especially exciting developments in structure-



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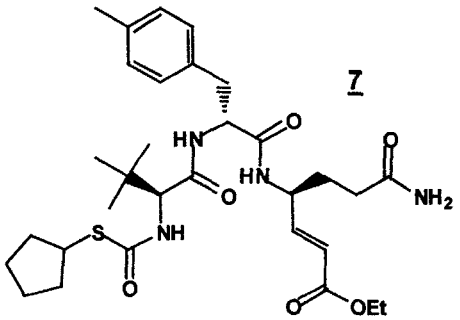

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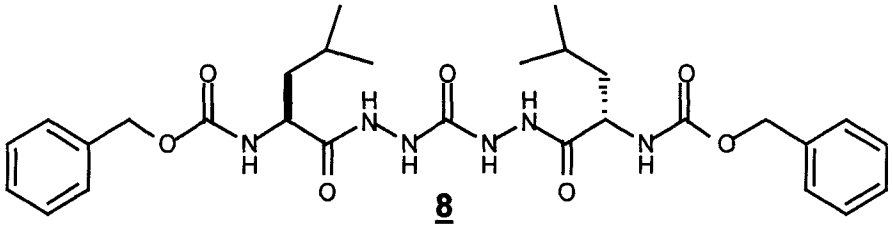
assisted design towards inhibitors of human rhinovirus 3C protease and cathepsin K. We direct interested readers to the following general reviews (66-68).

Human rhinovirus 3C protease - Early work (69) utilized the apo structure of human rhinovirus 3C protease to model peptide substrates into the active site to facilitate design of nonpeptide inhibitors. This led to the suggestion of 1,5-disubstituted isatin scaffolds since alpha-keto amides were known reversible inhibitors of cysteine and serine proteases. Further modeling studies helped choose functionality at the isatin 1- and 5- positions which might recognize the P1 and P2 subsites, respectively. Although some of these molecules had low nanomolar Ki's against the enzyme, they lacked in vitro antiviral potency. More recent work has focused on irreversible substrate-derived peptide inhibitors. Initial attempts at replacing the scissile amide bond by Michael acceptors followed by structural determination of such inhibitors led to optimization of prime side electrophiles (70). This analysis was performed in conjunction with a structure-driven systematic scan of optimal substitution in the P1 through P4 positions (71) and resulted in compound **7** with both rapid irreversible inhibition ($k_{obs}/(I)$ = 800,000 $M^{-1}s^{-1}$) and potent antiviral activity in cell culture (EC50 = 56 nM).

Cathepsin K - This target has been implicated in bone diseases such as osteoporosis. In a series of recent papers (72-76), iterative cycles of structure-based design, inhibitor synthesis and evaluation, and x-ray structure determination have been applied to the discovery of selective and quite novel peptidomimetic cathepsin K inhibitors, the prototype for which is the C2-symmetrical structure **8**. Later refinements



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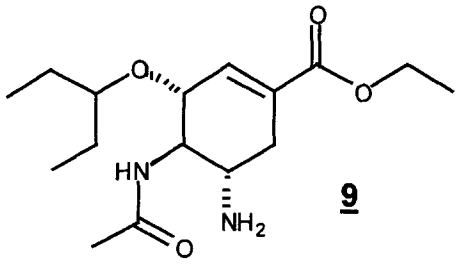
including the removal of the Cbz-leucine moiety ultimately lead to the identification of compounds with oral bioavailability (77).

Influenza neuraminidase - First generation inhibitors of neuraminidase (78, 79) are currently being optimized for oral bioavailability (80, 81). Structure-based design work was based on novel carbocycles as transition-state based inhibitors of influenza neuraminidase (82-85). A dihydropyran ring was replaced with a cyclohexene scaffold based upon analysis of crystallographic complexes of neuraminidase with sialic acid. The catalytic mechanism of sialic acid cleavage from glycoconjugates proceeds through a flat C2 carbonium cation transition state, which, it was reasoned, the cyclohexene scaffold would more closely resemble. Further analysis of crystallographic complexes suggested filling a large hydrophobic pocket in the region corresponding to the glycerol subsite of bound sialic acid. Lipophilic modifications were made to optimize hydrophobic_interactions with the enzyme. Optimization of potency and pharmacokinetic profile identified a clinical candidate with good activity against both influenza A and B strains. GS4104 (**9**) represents the first orally bioavailable anti-influenza compound to enter clinical trials.



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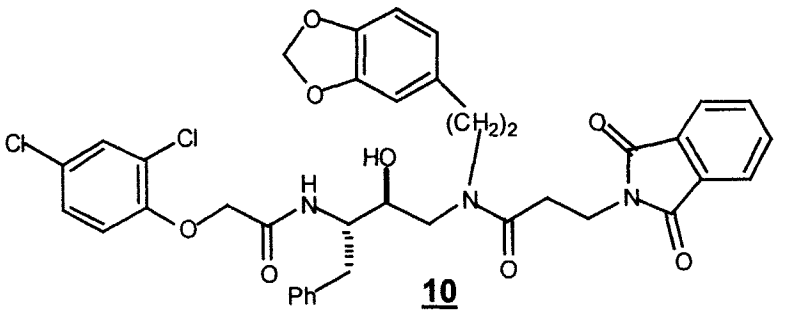
ADVANCES IN TECHNOLOGY RELEVANT TO STRUCTURE-BASED DESIGN

Methodology for structure-based library design - A number of research groups have described methodology to perform structure-assisted design of combinatorial libraries. Several recent reviews are available (7-9).

The design of cathepsin D inhibitors - The crystal structure of the aspartyl protease cathepsin D complexed with pepstatin was used to select building blocks for a solid phase hydroxyethylamine-based library (86). After filtering to remove high MW and synthetically-incompatible monomers, several starting conformations of the scaffold were chosen to place side chains in situ. The top scoring components from each of the independent starting positions were merged. These top scorers were then clustered to maximize diversity of monomers selected for synthesis. For comparative purposes, a "diverse" library starting from the same monomer set was designed based upon simple clustering. At a concentration of 1 μM there were 67 molecules (out of 1000) with 50% inhibition for the structure-based (directed) design and 26 (out of 1000) from the diverse library design. At 100 nM there were 7 molecules (out of 1000) with 50% inhibition for the directed design set and 1 from the diverse design set. This suggests that the structure-focused library design was much more powerful than a random approach. A second generation library based upon the most active compounds in the first directed library was pursued and this led to some low nanomolar inhibitors of cathepsin D, the most potent of which (**10**, Ki = 9 nM) is shown.



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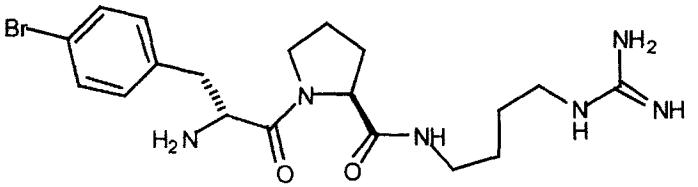
Design of nanomolar thrombin inhibitors - L-proline was used as a template with two points of substituent attachment for carboxylic acids and amines (87). Beginning with 4000 amines and 9000 carboxylic acids, the lists were filtered by a variety of property and functionality screens followed by independent evaluation of each substituent at each position. The remaining monomers in the receptor site were studied using an empirical scoring function to rank monomers. After this exercise there were 400 amines and 900 acids which were clustered to give 8 amines and 9 acids. This 72-compound library was fully enumerated and docked, leading to the selection of 30 molecules for synthesis. The most potent inhibitor pBr-d-Phe-Pro-Agmatine (11 , Ki=40 nM) closely resembled known thrombin inhibitors.



Structure and thermodynamics - Over the past few years there has been a dramatic increase in publication of papers discussing the use of direct thermodynamic measurements (usually from calorimetry) in drug design (88-96). The implications for drug design are especially interesting when combined with structural studies. We direct interested readers to the above references for some of the more recent and informative studies in this area.

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Chapter 30. Using Mass Spectrometry to Determine ADME Properties in Drug Discovery

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INSTRUMENTATION

Liquid chromatography/mass spectrometry (LC/MS) has become a common analytical tool for drug metabolism and pharmacokinetic studies. For several decades, mass spectrometry has been used to elucidate the structure of drug metabolites. Much work was accomplished by labor-intensive isolation of metabolites and direct MS analysis. The commercial development of gas chromatography coupled with mass spectrometry (GC/MS) afforded a highly sensitive and selective instrument for quantitative or qualitative analysis. The limitations of gas chromatography inspired the development of LC/MS interfaces. Over the past decade, the use of an atmospheric pressure interface (*electrospray* and *atmospheric pressure chemical* ionization) has proven to be the most rugged and sensitive interface for HPLC (1-3). When coupled with mass spectrometers capable of performing MS/MS analysis, both highly selective detection and informative spectroscopic data can be obtained.

The use of LC/MS/MS to identify drug metabolites from biological fluids or tissues with minimum or no sample extraction is straightforward. The most commonly used mass spectrometers have been triple quadrupoles (4) and ion traps (5). Ion traps are increasingly being used, due to their low cost, full scan sensitivity, and ability to perform multiple stage of mass (MS^n) analyses. In one application, data-dependent *scanning yielded complete fragmentation pathways for* glyburide metabolites in liver microsomes (6). Improvements in software involve intelligent instrument control (data-dependent scanning) and expert spectroscopic interpretation. Ion traps are also capable of providing higher resolution analysis, however, mass accuracy has not been achieved (7). Increasingly, time-of-flight (TOF) mass spectrometers are being used, either directly or with prior mass-analysis (Q-TOF). Time-of-flight analyzers using reflection optics afford moderate (RP = 4,000 to 12,000) mass resolution and are less expensive and easier to operate than magnetic double focusing mass spectrometers (8). When combined with quadrupole mass analysis, high resolution and accurate mass measurement of product ions aids in identifying complex mixtures separated by HPLC (9). Problems adapting higher performance atmospheric pressure *interfaces to magnetic* mass spectrometers were overcome in the early 1990's, and commercial instrumentation affords high resolution accurate mass measurement and MS/MS capability. Hybrid instrumentation coupling double focusing magnetic sector instrumentation with quadrupole, ion trap and TOF analyzers are commercially available. Due to their increased cost and complexity, hybrid instruments are less commonly used for metabolism studies (10).

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Mass spectrometry is often incapable of distinguishing more subtle differences in structure in a predictable manner. The addition of NMR to the analysis of metabolites affords more complete characterization. Commercial developments in LC/NMR (11) have allowed other spectroscopic instruments to be coupled with LC/MS/MS for metabolite identification (12). The addition of tools that resolve minor differences in structure, such as isomers or phase II conjugates, is essential to fully characterize important metabolites (13). NMR is several orders of magnitude less sensitive than mass spectrometry and is generally reserved for more complex structural assignments.

Prior to pre-clinical development, metabolite profiling is generally performed using MS rather than radiochromatographic detection. MS detection has better selectivity, and often exceeds the sensitivity of radiochromatographic detection. Unfortunately, the wide variation in ionization efficiency amongst metabolites makes it difficult to assess relative exposure from reconstructed ion chromatograms. Radiochromatographic detection allows quantitation of metabolites, thereby affording exposure and disposition information. However, synthesis of a radiolabeled compound is usually time and labor-intensive and is often reserved for pre-clinical excretion balance and metabolite profiling studies.

Alternative methods to radiochemical quantitation include ^{19}F-NMR (14), element-selective chemiluminescent detection (15) and chemical reaction interface for mass spectrometry (CRIMS) (16). All provide better guidance in assessing the relative proportions of individual components than ultraviolet (UV), fluorescence (FL) or MS detection. The detection of fluorine is highly specific in biological fluids. Unfortunately, NMR is relatively insensitive, expensive and often incapable of spectrally resolving complex mixtures of metabolites. The limited spectral dispersion can be overcome by coupling with HPLC, but generally requires stop-flow detection and lengthy analysis times to enhance sensitivity. Both nitrogen and sulfur selective chemilluminescent detectors are commercially available for HPLC but neither has been used for metabolism studies. CRIMS uses a microwave-induced helium plasma to dissociate molecules into atoms, react them with various gases, and monitor the stable reaction products using mass spectrometry. CRIMS provides both high sensitivity and selective detection. It has been coupled with HPLC and used for metabolite profiling studies (17-18). However, both CRIMS and element-selective detectors suffer when analyzing complex plasma or urine samples containing a high endogenous background of the element.

Metabolic toxicity or clearance issues are greatly aided by knowledge of the exact structure of the metabolite and its physiological concentration. While mass spectrometry has played an important role in defining metabolic liabilities of discovery candidates, even better means to rapidly determine this information on discovery candidates will help to define critical metabolic pathways.

In subsequent pre-clinical or clinical development, excretion balance, metabolite profiling and ^{32}P post-labeling

studies are aided by accelerator mass spectrometry (AMS). This ultra-sensitive method for the direct determination of ^{14}C, ^{3}H or ^{32}P atoms in biological fluids or tissues requires considerable care in sample preparation and expensive instrumentation (19). However, it affords orders of magnitude better sensitivity than decay-counting techniques. Dose requirements are considerably reduced, making possible studies previously restricted by radiation exposure (20).

HIGH-THROUGHPUT *IN VITRO* SCREENING

In vitro absorption studies performed using cultured cells such as Caco-2 or MDCK can be adapted to automation. When not limited by cell growth, analytical resources limit throughput. LC/MS is the tool of choice for fast method development and highly sensitive detection (21). Recognizing the need for rugged ion sources capable of withstanding prolonged exposure to the high salt media used in Caco-2 studies, instrument vendors have developed electrospray sources with an off-axis interface (22). High throughput absorption or metabolism studies often rely upon direct injection of high salt buffers into the LC/MS. Ion sources that are impervious to the high salt background are preferred for *in vitro* studies.

In vitro studies may be performed at various stages of the discovery screening and selection process. Studies to select candidates that are stable in liver hepatocytes or sub-cellular fractions such as microsomes and S9 have been aided by establishing systematic LC/MS procedures (23). Preliminary stability assays or intrinsic clearance determinations are often coupled with qualitative analysis to identify metabolic 'soft spots'. However, validation of *in vitro* studies against *in vivo* results should be done before relying upon metabolic stability as a primary screen.

A pulsed ultrafiltration mass spectrometric method to identify metabolites generated by rat liver microsomes can be used to rapidly screen drug candidates (24). Both stability and structural information was quickly obtained using MS/MS. The elimination of chromatographic analyses afforded a high throughput method for metabolite screening.

Drug-drug interaction studies, once only considered for later drug development, are increasingly being done in discovery. Many traditional assays are time-consuming, relying upon HPLC and UV, FL or radiochemical detection of specific cytochrome P450 probe substrates. Automated fluorescence assays using selective probes and recombinant P450 isozymes are a higher throughput approach but rely upon the use of recombinant P450 isozymes (25). An alternative method employs LC/MS/MS assays of cytochrome P450 probe substrates in microsomes (26). Probe substrates for seven isozymes, including 2D6 and 3A4, were analyzed under various conditions. The use of MS detection affords much greater selectivity in detection than fluorescence and better sensitivity and throughput than chromatographic assays.

IN VIVO PHARMACOKINETIC SCREENING

Many *in vitro* approaches use LC/MS with fast isocratic or gradient chromatographic analyses. Throughput is driven by chromatographic speed. Unlike most HPLC detectors, mass spectrometric detection yields chromatograms that are often limited by electronic noise rather then chemical noise. A second stage of MS detection (MS/MS) can often provide a chromatogram that is devoid of other peaks. Many analysts reduce chromatographic analysis times or avoid separations entirely. For the analysis of sensitive compounds in clean matrices, this approach will afford a cost-effective analytical method with limited risk (27). For the analysis of complex samples without extraction, this approach can result in severe matrix effects (28). For *in vitro* studies, matrix effects are often self-correcting as controls are prepared from the same matrix and analyzed side-by-side with samples. A loss in sensitivity is observed, but is compensated by a similar loss in response of the control. Due to its greater variability, less favorable results are obtained from *in vivo* studies.

Rather than relying upon fast analytical run times, a different approach to improving throughput in pharmacokinetic studies uses the full resolving power of LC/MS/MS to analyze more complex samples. The simultaneous dosing of compounds in animals arose in response to demands placed upon drug metabolism from combinatory chemistry (29). Parallel synthesis of potential drug candidates required parallel pharmacokinetic studies. For those bold enough to dose pools, both the animal and analytical throughput improved. Others preferred not to risk drug-drug interactions, dosing separately in animals and pooling samples for analysis (30).

The procedure of simultaneously dosing 'N' compounds was originally called N-in-1, although simultaneous compound dosing, cocktail or cassette dosing have also been used. This polypharmacy approach to improve throughput is practiced differently, largely dependent upon chemistry and drug metabolism resources.

The advent of combinatorial chemistry has overwhelmed the capacity of animal and analytical resources. When chemistry is completed before any drug metabolism studies are undertaken, the pharmacokinetic data is used only for selecting the best candidate(s) and does not aid new synthesis. If new candidates are not subjected to other primary pharmacology or biological screens, it is often necessary to screen libraries for physicochemical or *in vitro* properties (31).

Organizations that do not screen compounds and desire pharmacokinetic data must decide what risks they are willing to accept. Groups with limited animal resources may decide to dose large numbers of structurally diverse compounds in pools. In any therapeutic program, it is important to decide how large the pool (N) should be. There is not one answer; it is inferred from study results. It is best to start small and examine the

data closely for possible interactions before increasing 'N' (32). An efficient grouping is 10-25 compounds per pool.

In an interactive approach, less than 50 compounds are made available within the same therapeutic area. Based upon their ability to be formulated and measured with minimum potential for interaction, 2-3 pools may be selected for dosing. Pharmacokinetic data is returned quickly, as the information helps to define new synthesis. A complete profile of pharmacokinetic (Cl, V_d, $t_{1/2}$, F) data from IV and PO dosing, protein binding and target (CSF, synovial fluid) concentrations are returned within 4-6 weeks of receipt of the compounds.

In all cases, one should use as low a dose as possible. Within a structurally related series of chemicals, a safe dose is derived by N-fold dilution of a dose for the positive control that yielded proportional pharmacokinetics in the species under study. A positive control, a related compound with known pharmacokinetics, serves to gauge the quality of data. Repeat discrete studies validate the N-in-1 results (29).

Obvious analytical problems, such as the detection of a pool metabolite or overlapping isotopes must be eliminated or resolved. Otherwise, it will be difficult to interpret analytical problems from potential drug-drug interactions. Isomers, isobaric

compounds and suspected metabolites should be excluded from the pool. False positives are indicated later, but reduce screening efficiency. Complex formulations whose components affect only the MS response of individually co-eluting components can result in false negatives and should be avoided.

There are numerous analytical issues that must be addressed before dosing. Generic, non-selective extraction procedures should be developed for the class of compounds under study. The extraction procedure should recover all analytes in the pool with a reasonable degree of enrichment. Preferably, the procedure can be automated (33-34). A high-resolution separation helps to minimize matrix effects. Gradient elution provides added sensitivity by trace enrichment focussing. Analysis time and batch size is generally not limiting. The selection of appropriate internal standards(s) is critical. Usually, many are available.

Selective detection should be achieved using MS/MS. The diversity in molecular weight is not usually sufficient to make full use of the operating range of the mass spectrometer. Considering the presence of plasma metabolites for a large N-in-1 study of limited MW diversity, analysis using a single quadrupole LC/MS would be risky. Most work has been done using triple quadrupole or ion trap mass spectrometers. Triple quadrupoles are preferred for their sensitivity, precision and accuracy. Ion traps can be used and are capable of further enhancing resolution by MS^n analysis. However, the low ion abundance in these experiments often precludes quantitation using more than MS^2. Separation in the second mass/charge dimension may not also be as useful as first thought since similar structures have similar fragments. High-resolution mass

analysis is also possible, but would require differences in the mass sufficiency or deficiency of isobaric ions to be effective. High resolution has not been used for N-in-1 analyses but may be useful for chemically diverse pools. In all cases, there is no substitute for sensitivity. The financial success of triple quadrupole mass spectrometers with improved ion transmission attest to this need.

Analytical precision and accuracy are generally criteria that are measured by validation prior to sample analysis. Target values are fixed and assays either meet or fail acceptance. For discovery pharmacokinetic screening, there are no rules. For a 20-in-1 analysis, acceptance at a 95% confidence interval still predicts that 1 in 20 will fail. Multiple component methods have enhanced probability of failure with increasing 'N'.

Those individuals exploiting sparse sampling or pooling approaches may perform no validation, choosing to estimate concentrations by bracketing with standards. Winners are repeated with greater accuracy and precision. Groups obtaining more complete pharmacokinetic data in higher species will generally execute a validation of the method prior to analysis of study samples (35). Study results for each compound are often judged along with their acceptance criteria (32). Greater differences in pharmacokinetics allow less precise or accurate data to be used with little impact. Often, failures may be repeated in the next pool.

When a larger risk is acceptable, a significant increase in throughput is possible. As many as 90 compounds have been dosed in a single animal (36). A limited number of concentrations at later times were determined and used to estimate half-lives. Complete pharmacokinetic profiles were not determined. In general, higher concentrations than those seen in smaller N-in-1 or discrete studies were noted. However, rapidly eliminated compounds were identified. A large 'N' study may be appropriate when one is willing to accept a greater risk or simply wants to establish a relationship between structure and pharmacokinetics (37).

The data returned from IV N-in-1 studies may be better than from oral studies. Many factors can negatively affect oral studies, including inhibition of intestinal transporters (38) or first-pass metabolism (39) and variations in individual formulation solubility. A high concentration in a localized area can saturate enzymatic processes involved in the distribution or metabolism of compound. This may be due to the inhibition of clearance masking first-pass metabolism or the inhibition of active transporters, such as P-glycoprotein in the intestine. Oral doses can more easily yield saturable concentrations resulting in inhibition of one component by another (false positive). Dosing suspensions may be composites of compounds with varying degrees of solubility. Compounds in suspension may exhibit dissolution rate-limited adsorption and have poorer bioavailability than ones in solution (false negative). An individual component's solubility in a mixture is complex and absorption can be affected differently by food. Dissolution in bile salts can help to guide formulation

development (40). IV dosing requires a soluble formulation, and drug is quickly diluted into the systemic circulation.

In many higher species, animal costs eclipse analytical costs. Expenses associated with formulation development and pharmacokinetic analysis are relatively small. Costs decrease with increasing 'N'. Obtaining pharmacokinetic data on as many candidates as possible, while avoiding analytical errors or critical drug-drug interactions is the key to a successful screening program.

POOLING SAMPLES FOR GREATER ANALYTICAL THROUGHPUT

Organizations with limited analytical resources may use different pooling approaches to minimize analytical demands. Higher concentrations can also be dosed without fear of interaction, placing fewer demands upon the analyst.

There are several means to reduce the number of samples for analysis. The most common is to pool samples from different animals. Information regarding inter-animal variability is lost, but sample analysis is reduced. This approach is also used to improve detection sensitivity when the analysis is not limited by chemical noise.

A second means of reducing the number of samples is to pool them from different animals dosed with different drug candidates (30, 41). This type of pooling adds inter-animal variability into the analysis but avoids drug-drug interaction. This sample is similar to the one derived from an N-in-1 study. Provided it can be enriched during extraction and the individual compounds were dosed at full potency, a similar gain in detection sensitivity may be possible for analytes not limited by chemical noise.

A third means is to pool samples from different collection times (42-43), affording an average AUC for the collection interval. This approach reduces analysis to a single sample and can be used to estimate oral exposure. Time-weighting the sample concentration is needed to obtain better estimates.

Pooling samples from different times to establish an average AUC can considerably reduce analytical costs. Organizations exploiting this approach are typically less rigorous in their analytical methods, accepting results as a bioavailability screen for rank order. Pooling has most value for less expensive rodent studies. In more expensive animal (primate, dog) studies, a more complete set of pharmacokinetic data is usually desired.

Combinations of each approach can further reduce the number of samples to be analyzed. Consider a study in which 10

compounds are dosed individually (four animals per dose group) and 10 samples are collected (10 x 4 x 10 = 400 total samples). By using every pooling approach, four hundred samples can be reduced to a single sample for a time-averaged AUC determination of 10 compounds.

An advantage of pooling approaches is that it affords samples for metabolite profiling and a better correlation with pharmacodynamics. While it is possible to correlate N-in-1 pharmacokinetics with discrete pharmacodynamics, inter-animal variability can affect the results.

Where animal resources are not used, sample pooling may be the preferred approach. Analytical cost is then dominant, and competitive binding or inhibition problems are avoided. This includes protein binding and *in vitro* (absorption, metabolism) screening.

Cocktail approaches to protein binding have been investigated (44). A good correlation existed between the free-fractions determined in the cocktail with discrete data. A wide range (0.6%-27.1%) of free fractions were observed for the 44 compounds that were tested. However, none exhibited nonspecific binding to the ultrafiltration device.

In an absorption study, six arbitrary mixtures containing 24 diverse peptoids were evaluated in Caco-2 (45). 'Aggregate' apparent permeability coefficients were determined to rank the effect of various template structures. The study demonstrated that data obtained from pools was comparable to the individual determination and could be used to study structure-transport relationships. More data from cocktail protein binding and absorption studies is needed to determine its benefits and risks.

SUMMARY

LC/MS has enabled drug metabolism and pharmacokinetic studies, previously reserved for later development, to be executed in discovery. Metabolite profiling and identification, *in vitro* clearance and inhibition measurements, absorption and pharmacokinetic studies are aided using LC/MS/MS. When coupled with automation

and software developments, screening hundreds or even thousands of compounds within a therapeutic program is possible.

The simultaneous dosing and analysis of multiple components can be used to rapidly obtain pharmacokinetic data. The alternative, sample pooling, maintains analytical throughput but requires extensive animal facilities. Resources determine its use and, like any high throughput screen, there is a risk vs. benefit. The benefit is easily calculated when there are no drug-drug interactions or false positives. False negatives are rare, but can result in the loss of good compounds. Good analytical data is the foundation for efficient screening.

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SECTION VII. TRENDS AND PERSPECTIVES

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Chapter 31. To Market, To Market - 1998

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In 1998, the number of new therapeutic chemical entities (NCEs) introduced into the world market for the first time was 27 (1), the fewest during the nineties. This represented a marked decrease from 39 in 1997 (2), 38 in 1996 (3), 35 in 1995 (4), 44 in the record-breaking year of 1994 (5), 43 in 1993 (6), 36 in 1992 (7) and 1991 (8). However interestingly, there was a rather large number of new biological entities (NBEs) approved and launched in 1998 : among the new active substances, about one NBE could be identified for three NCEs, the tendancy for this ratio being to significantly increase from year to year. Some NBE representatives introduced in 1998 will be briefly reviewed in the last part of this introduction.

With 16 NCE launches, the US was by far the most common first market for the new introductions. Switzerland was second with 4 NCEs launched, followed by Germany and Japan with 3, then Finland and Mexico with 2 new introductions. Finally, several countries, Australia, New Zealand, Netherlands, Sweden came with one NCE introduced. Regarding the *originators* of NCEs first launched in 1998, once again the US ranked first with 13, second trailed Germany with 5 NCEs. Japan was third with 3, then Switzerland and France with 2 each. UK and Finland came next with 1 original molecule each. One pharmaceutical company, Merck & Co. dominated with 4 NCEs originating there, followed by Pfizer, Roche and HMR with 2 each.

Among the NCEs launched for the first time in 1998, several were the first in class or with a novel mechanism of action.Viagra (Sildenafil), often proclaimed as the new active molecule of the year, was introduced as an orally-active potent inhibitor of PDE5 isozymes for the treatment of male erectile dysfunction. Vitravene (Fomivirsen) is a first-generation cytomegalovirus replication inhibitor and the only agent based on antisense technology to have obtained a marketing approval so far. Evista (Raloxifene), the first selective oestrogen receptor modulator, was launched for the prevention of postmenopausal osteoporosis. Arava (Leflunomide) was indicated for the treatment of rheumatoid arthritis, being the first and only drug indicated to date for delaying joint damage in RA.

Xenical (Orlistat) is another major high profile launch of last year ; this potent inhibitor of gastrointestinal lipases prevents the absorption of dietary fat and was introduced for the long-term treatment of obesity.

With the introduction of several new products, the class of agents affecting blood coagulation was one of the most active : Plavix (Clopidogrel) inhibits ADP-induced platelet aggregation and is indicated for the preventive management of secondary ischemic events in patients with established peripheral arterial disease. Aggrastat (Tirofiban) is a non peptidic antagonist of platelet GPIIb/IIIa receptor and prevents arterial thrombosis, being indicated for patients with unstable angina.

Following the withdrawal of Tasmar, the first COMT inhibitor marketed in 1997, a second one, Comtess (Entacapone) made its debut last year for the treatment of Parkinson's disease in combination with levodopa and a dopa carboxylase inhibitor.

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The only anti-HIV introduced in 1998 was Sustiva (Efavirenz), a non-nucleoside reverse transcriptase inhibitor ; it was shown that its antiretroviral combination with two nucleosides was more effective and better tolerated than a combination using a protease inhibitor.

Many new biological entities (NBEs), generally biotechnology-based products, were marketed in 1998. By way of examples, the following list of recombinant proteins can be mentioned :

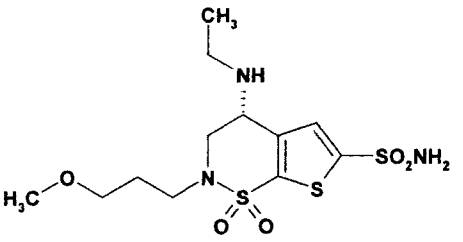
- Becaplermin (Chiron) : Recombinant human platalet-derived growth factor (rhPDGF) for the treatment of skin ulcers, particularly foot ulcers, in diabetic patients.
- Etanercept (Immunex) : soluble recombinant human p75 TNF receptor and human IgGI Fc portion fusion protein produced in a mammalian cell expression system ; approved as a second-line agent for the treatment of rheumatoid arthritis and other inflammatory conditions, particularly those mediated by TNF.
- Lepirudin (Hoechst Marion Roussel) : biosynthetic recombinant hirudin from yeast cells, slightly different from natural hirudin, acting as a highly specific direct inhibitor of thrombin ; indicated for providing anticoagulation in those patients having heparin-induced thrombocytopenia and in thromboembolic disease.
- Monteplase (Eisai) : modified human tissue plasminogen activator obtained by use of recombinant DNA technology ; developed and marketed for the treatment of acute myocardial infarction.
- Oprelvekin (Genetics Institute) : thrombopoietic growth factor recombinant human interleukin-11 for use in preventing recurrent severe thrombocytopenia and reducing the need for frequent transfusions following myelosuppressive high-dose chemotherapy.
- Thyrotropin Alfa (Genzyme) : recombinant human thyroid stimulating hormone for use in follow-up screening of patients who have been treated for thyroid cancer.

Also, it is noteworthy that several chimeric monoclonal antibodies were launched last year :

- Basiliximab (Novartis) : immunosuppressant that blocks the IL-2 receptor, a protein implicated in the proliferation of T-lymphocytes ; indicated in the prophylaxis of acute rejection episodes in renal transplant recipients.
- Infliximab (Centocor) : monoclonal antibody that blocks the activity of TNF-alpha and consequently, opposes intestinal inflammation in patients with Crohn's disease.
- Palivizumab (Synagis) : humanized monoclonal antibody targeted to the F protein on the surface of respiratory syncytial virus (RSV), the leading cause of pneumonia and other severe infectious diseases in infants.
- Trastuxumab (Genentech) : humanized monoclonal antibody for the treatment of epidermal growth factor receptor-2 overexpressing metastatic breast cancer.

Brinzolamide (Antiglaucoma) (9 – 14)

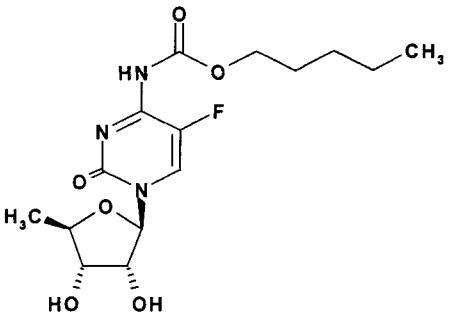
Country of Origin : US
Originator : Alcon
First Introduction : US
Introduced by : Alcon
Trade Name : Azopt
CAS Registry No : 138890-62-7
Molecular Weight : 383.50



Brinzolamide was introduced as Azopt in the US for the treatment of elevated intraocular pressure (IOP) in patients with ocular hypertension or open-angle glaucoma. Brinzolamide is a potent inhibitor of human carbonic anhydrase II lowering IOP after topical administration . It is the second of this class after dorzolamide (1995). Brinzolamide can be prepared in an eight-step sequence from 3-acetyl-2,5-dichlorothiophene. In patients with primary open-angle glaucoma or ocular hypertension, brinzolamide produced significant reductions in IOP and showed less ocular discomfort than dorzolamide.

Capecitabine (Antineoplastic) (15 – 20)

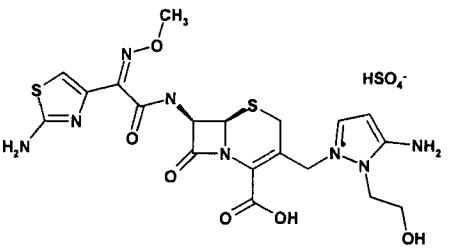
Country of Origin : Switzerland
Originator : Roche
First Introduction : Switzerland
Introduced by : Roche
Trade Name : Xeloda
CAS Registry No : 154661-50-9
Molecular Weight : 359.36



Capecitabine is a new oral fluoropyrimidine carbamate for patients with advanced neoplastic disease, approved as Xeloda for the treatment of refractory metastatic breast cancer after failure on Paclitaxel and an anthracycline-based chemotherapy regimen ; it is a prodrug of doxifluridine (5-fluorouracil ; 5-FU) activated by a cascade of 3 enzymes concentrated in human liver and cancer tissue, resulting in the selective release of 5-FU at the tumor site and offering a prolonged tumour exposure to 5-FU. Oral Capecitabine passes intact through the intestinal mucosa, is converted first by carboxylesterase to 5'-deoxy-5-fluorocytidine in the liver, then by cytidine deaminase to 5'-deoxy-5-fluorouridine in the liver and tumour tissues and finally by thymidine phosphorylase to 5-FU in tumors. Therefore, Xeloda is much safer and more effective than 5-FU (for example, in the HCT116 human colon cancer and the MX-1 breast cancer xenograft models).

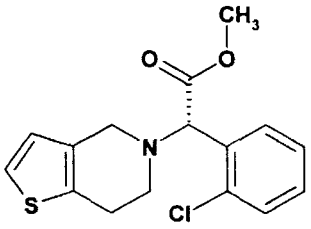
Cefoselis (Antibiotic) (21 – 27)

Country of Origin : Japan
Originator : Fujisawa
First Introduction : Japan
Introduced by : Fujisawa
Trade Name : Wincef
CAS Registry No : 122841-12-7
Molecular Weight : 620.63



Cefoselis is a new fourth-generation cephalosporin and was launched in Japan as a parenteral antibiotic for a variety of infections including *Staphylococcus aureus* (particularly the methicillin-resistant MRSA) and *Pseudomonas aeruginosa*. It can be prepared in 3 related ways, all using 2-pyrazolomethyl-3-cephem-4-carboxylic as the key intermediate. In preclinical studies, Cefolesis had better MIC50s than Ceftazidime against *Streptococcus pneumoniae* or *Staphylococcus aureus* (methicillin-sensitive MSSA or resistant MRSA) and showed significant antibacterial activity against *Citrobacter* and *Enterobacter*.
Cefolesis is well tolerated in clinical studies, being eliminated mainly via glomerular filtration in humans.

Clopidogrel Hydrogensulfate (28 – 35)
(Antithrombotic)
Country of Origin : France
Originator : Sanofi
First Introduction : US
Introduced by : Sanofi, Bristol-Myers Squibb
Trade Name : Plavix, Iscover
CAS Registry No : 113665-84-2
Molecular Weight : 419.89

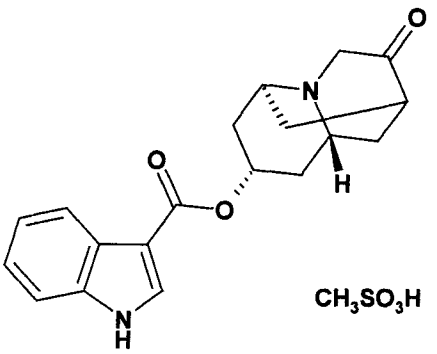


.H2SO4

Clopidogrel was launched in the US as a potent inhibitor of platelet aggregation for the preventive management of secondary ischemic events, including MI, stroke and vascular deaths. Clopidogrel can be synthesized in 4 steps (including an optical resolution to the S active enantiomer) from 2-(2-chlorophenyl)-glycine, the key step being the cyclization to thienopyridine with formaldehyde and acetic acid. Clopidogrel belongs to the original chemical class of Ticlopidine, but shows fewer side effects (in particular, bone-marrow-suppressing effects) at the dosage generally used. Like Ticlopidine, it is an Adenosine diphosphate (ADP) antagonist acting at the purinergic P2y receptor. In in vivo experiments with rabbits, Clopidogrel shows a maximal antiaggregant effect at 20mg/kg po, reducing adhesion of platelets to the vascular subendothelium ; moreover, it reduces myointimal thickening occuring after endothelial injury of rat carotid artery. Clopigrel does not affect platelet aggregation in vitro ; actually, its in vivo activity is highly dependent on hepatic metabolism. The results of a CAPRIE trial (Clopidogrel versus Aspirin in patients at risk of ischemic events) demonstrated that Clopidogrel was well tolerated and more effective than aspirin.

Dolasetron Mesylate (36 – 42)
(Antiemetic)

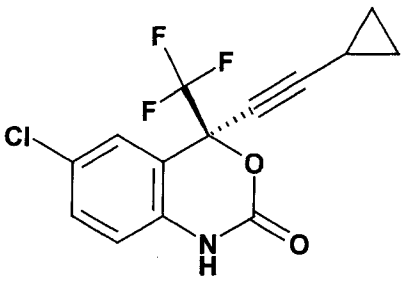
Country of Origin : Germany
Originator : Hoechst Marion Roussel
First Introduction : Australia
Introduced by : Hoechst Marion Roussel
Trade Name : Anzemet
CAS Registry No : 115956-13-3
Molecular Weight : 438.49



Dolasetron was launched as Anzemet in Australia and the US for the prevention of nausea and vomiting in chemotherapy patients. It is a highly potent and very selective antagonist of 5-HT3 receptors ; it is the sixth in this class of compounds to be marketed for the treatment of chemotherapy-induced emesis. The last two approved in this class were Nazasetron (1994) and Ramosetron (1996). Anzemet was prepared by a seven step sequence from a cyclopentenecarboxylic ester via a Robinson-Schöpf cyclisation of a dialdehyde into a key 9-azabicyclo[3.3.1]nonan-3-one. In a clinical study with 164 cancer patients treated with Dolasetron mesylate prior to Cisplatin, single doses of 10-50 mg achieved major control of nausea and emesis in 73% of subjects and were well tolerated. Results from pharmacokinetic studies in humans showed that the clinical effects and duration of action seem to be due mainly to a major plasma metabolite rapidly formed and very potent itself, the (+) enantiomeric alcohol obtained by enzymatic reduction of the cyclic ketone.

Efavirenz (Antiviral for AIDS) (43 – 48)

Country of Origin : US
Originator : Merck & Co.
First Introduction : US
Introduced by : DuPont Pharm.
Trade Name : Sustiva
CAS Registry No : 154598-52-4
Molecular Weight : 315.68

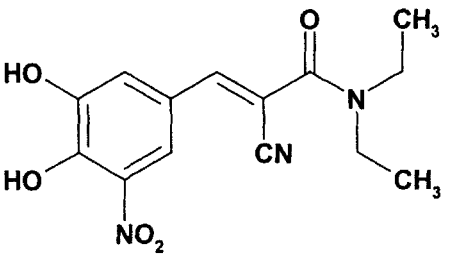


Efavirenz was launched as Sustiva in the US for the treatment of infection by HIV, the virus causing AIDS, in combination with other anti-retroviral agents.
Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) belonging to the 3,1-benzoxazin-2-one chemical class. It is the third non-nucleoside reverse transcriptase inhibitor to have been launched to date, after Nevirapine (1996) and Delavirdine (1997), increasing the
arsenal of anti-HIV drugs for treating infected patients in dual or triple combination with nucleoside or other non-nucleoside RTIs, or protease inhibitors.

Efavirenz can be obtained by two related ways of six steps from 4-chloroaniline ; one of them is based on asymmetric synthesis by enantioselective addition of an acetylide to a trifluoroacetophenone. The anti-HIV activity of Efavirenz was demonstrated against most wild-type and clinical strains of HIV-1, including those with the most frequently observed mutations. Efavirenz has a better pharmacokinetic profile when compared with the preceding drugs of this class ; in particular, in a long-term experiment conducted in cynomolgus monkeys, Efavirenz was shown to easily cross the blood brain barrier leading to an increase of the antiviral concentration in cerebrospinal fluid.

Entacapone (Parkinson's disease) (49 – 56)

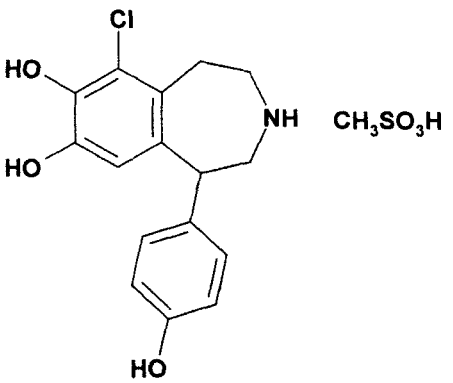
Country of Origin : Finland
Originator : Orion Pharma
First Introduction : Finland, Germany, Sweden
Introduced by : Orion Pharma
Trade Name : Comtess
CAS Registry No : 130929-57-6
Molecular Weight : 305.29



Entacapone was introduced in Finland, Germany and Sweden as an adjunctive treatment with L-dopa in Parkinson's disease. Entacapone is the second drug in its class to reach the market ; it can be obtained by basecatalyzed condensation of the corresponding benzaldehyde with a cyanoacetamide. Entacapone is a highly selective and orally-active catechol-O-methyltransferase (COMT) inhibitor ; by inhibiting metabolism of L-dopa when given as an adjuvant in patients with Parkinson's disease, Entacapone markedly prolongs the effects of L-dopa and improves its bioavailability. Results from clinical studies showed that 200mg/day Entacapone coadministered with L-dopa lowered the dose of the latter required to reduce fluctuations in motor performance

Fenoldopam Mesylate
(antihypertensive) (57 – 64)

Country of Origin : US
Originator : SmithKline Beecham
First Introduction : US
Introduced by : Neurex
Trade Name : Corlopam
CAS Registry No : 67227-57-0
Molecular Weight : 401.67

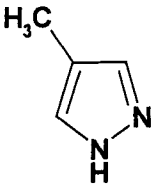


Fenoldopam, first approved in the Netherlands in 1992, ended by reaching its first market, the US, for the short-term management of severe

hypertension, including malignant hypertension, in the hospital setting. Fenoldopam can be prepared in 3 steps from the corresponding phenethylamine and aryloxiran, the pivotal step being the cyclisation in benzazepine in acidic medium. Fenoldopam is a potent dopamine D1 receptor agonist acting peripherally to produce systemic vasodilation.As it does not cross the blood-brain barrier, it does not exert significant central dopaminergic activity. Fenoldopam also interacts significantly with 5HT1c and 5HT2 receptors. In comparative trials with the most common drug used for this condition in Europe, Fenoldopam was found to be appreciably more potent than nifedipine. Furthermore, Fenoldopam is fast acting and maintains a long-lasting antihypertensive effect.

Fomepizole (Antidote) (65 – 70)

Country of Origin : US
Originator : Orphan Medical
First Introduction : US
Introduced by : Orphan/Cambridge
Trade Name : Antizol
CAS Registry No : 7554-65-6
Molecular Weight : 82.11



Fomepizole is the first drug indicated as an antidote for ethylene glycol poisoning (it has also shown promise as a treatment for methanol poisoning). Fomepizole is 4-methyl-1H-pyrazole active as a synthetic alcohol dehydrogenase inhibitor, blocking the formation of toxic ethylene glycol metabolites which are responsible for a severe metabolic acidosis and renal failure. In limited human studies, it reversed the toxicity of potentially lethal doses of ethylene glycol. The main urinary metabolite has been reported to be 4-carboxypyrazole by oxidation of the methyl group.Compared with the current treatment (ethanol), the duration of action is longer and the safety is improved, with no toxic effects reported.

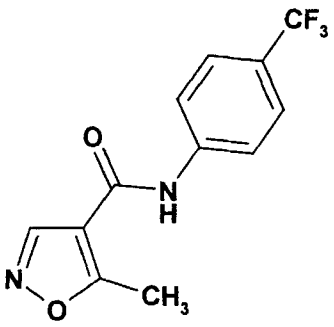
Fomivirsen Sodium (antiviral, treatment of CMV retinitis) (71 – 78)

Country of Origin : US
Originator : Isis Pharmaceuticals
First Introduction : US
Introduced by : Ciba Vision
Trade Name : Vitravene
CAS Registry No : 160369-77-7

Fomivirsen Sodium was launched as Vitravene in the US and is indicated for the local treatment of cytomegalovirus (CMV)-induced retinitis in AIDS patients. It consists of a phosphorothioate oligonucleotide (PS) comprised of a sequence of 21 nucleotides. Fomivirsen is synthesized using solid-phase phosphoramidite chemistry. Fomivirsen inhibits human CMV replication by a antisense mechanism : it targets an immediate early mRNA of cytomegalovirus, inhibiting the expression of two regulatory proteins. Fomivirsen was the most advanced first-generation PS drug candidate in human clinical trials and was the first agent based on antisense technology to reach the market. In normal human dermal fibroblast cells, Fomivirsen inhibited AD169 human CMV replication by more than 90%, at a concentration of 5μM ; it showed greater hCMV replication inhibitor properties than ganciclovir in several experimental models.

Leflunomide (Antiarthritic) (79 – 88)

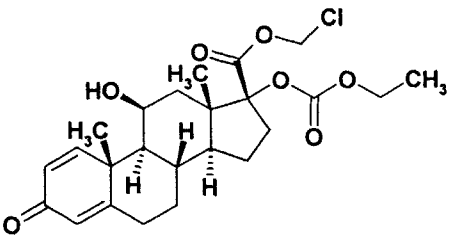
Country of Origin : Germany
Originator : Hoechst MarionRoussel
First Introduction : US
Introduced by : Hoechst Marion Roussel
Trade Name : Arava
CAS Registry No : 75706-12-6
Molecular Weight : 216.24



Leflunomide is an orally-available disease-modifying antirheumatic drug and was launched as Arava in the US for the treatment of rheumatoid arthritis (RA) ; it is the first and only drug to be indicated to slow down structural joint damage of RA, so addressing an unmet medical need.
Leflunomide is prepared in 3 steps from the appropriate acetoacetic anilide using a nitrile oxide- enamine cycloaddition reaction to assemble the isoxazole ring. Leflunomide is a prodrug, being extensively metabolized in vivo into the corresponding 2-cyano-3-hydroxy-2-butenamide resulting from fragmentation of the isoxazole ring. This cyanoenol is actually the active metabolite and several experiments in animals have demonstrated that after oral administration, substantial and sustained levels of this metabolite were delivered to the systemic circulation.
In vitro, Leflunomide's active metabolite inhibits dihydroorotate dehydrogenase, an enzyme involved in the biosynthesis of pyrimidine nucleotides, probably accounting for its immunosuppressive effect in vivo. Other mechanisms of action such as inhibition of tyrosine kinase and inhibition of responsiveness to interleukin-2 have been proposed. In diverse models of autoimmune or allergic diseases, Leflunomide showed efficacy both prophylactically and therapeutically.

Loteprednol Etabonate (89 – 94)
(Ophthalmic antiinflammatory, antiallergic)

Country of Origin : US
Originator : Pharmos
First Introduction : US
Introduced by : Bausch & Lomb
Trade Name : Lotemax (0.5%) ; Ali (0.2%)
CAS Registry No : 082034-46-6
Molecular Weight : 466.96

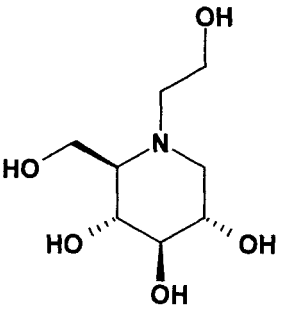


Loteprednol etabonate was introduced in the US as Lotemax (opththalmic suspension at 0.5%) for the treatment of steroid-responsive inflammatory conditions of the palpebral and bulbar conjunctiva, cornea and anterior segment of the ocular globe, and as Alrex (opththalmic suspension at 0.2%) for the symptomatic treatment of seasonal allergic conjunctivitis. Loteprednol etabonate is a novel soft corticosteroid with a superior efficacy and an improved safety profile compared to prior ophthalmic steroids due to its

metabolic lability and a fast enzymatic transformation to inactive metabolite. A combination of Lotemax with the antibiotic Tobramycin is currently under development.

Miglitol (Antidiabetic) (95 – 102)

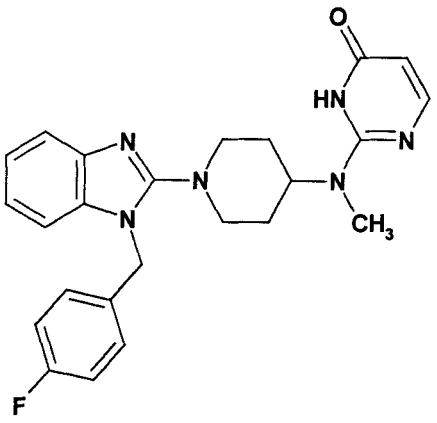
Country of Origin : Germany
Originator : Bayer
First Introduction : Germany
Introduced by : Sanofi
Trade Name : Diastabol
CAS Registry No : 72432-03-2
Molecular Weight : 207.23



Miglitol was introduced last year in Germany as an auxiliary treatment for non-insulin dependent diabetes (NIDDM). A simple two-step synthesis of Miglitol consists of converting 6-desoxy-6-aminosorbose into desoxynojirimycin by reductive cyclization, then selectively hydroxyethylating the cyclic nitrogen. Miglitol is a short-acting intestinal alpha-D-glucosidase inhibitor, reducing postprandial glucose excursions by helping to delay absorption of complex carbohydrates. Acarbose and voglibose are the previous compounds marketed in this class. At low doses, orally administered miglitol was shown to be rapidly and completely absorbed then removed by renal excretion.

Mizolastine (Antihistaminic) (103 – 108)

Country of Origin : France
Originator : Synthelabo
First Introduction : Germany, Switzerland
Introduced by : Galderma
Trade Name : Mizollen
CAS Registry No : 108612-45-9
Molecular Weight : 432.50

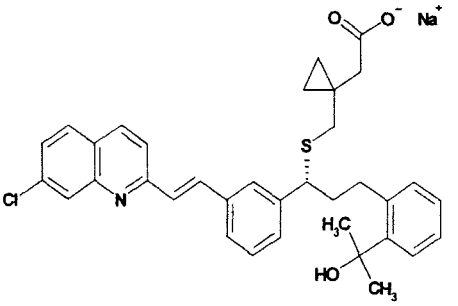


Mizolastine was marketed in Germany and Switzerland as Mizollen for the symptomatic relief of seasonal and perennial allergic rhinoconjunctivitis and urticaria. Mizolastine is a new long-acting, orally active antihistaminic agent with a rapid onset of action ; the two most recent H1 antagonists launched were fexofenadine, metabolite of terfenadine (Sepracor, 1996) and Olopatadine (Kyowa Hakko, 1997). Mizolastine can be prepared in 2 steps from 2-chloro 1-(4-fluorobenzyl)benzimidazole by successive condensations of appropriate

amine and thioether. Mizolastine selectively blocks the peripheral H1 receptors (but not the serotonergic, noradrenergic, muscarinic receptors) with a minimal occupancy of brain receptors, and therefore does not elicit any sedative effects. Moreover, Mizolastine does not produce cardiac rhythm disorders which have been associated with certain non-sedating antihistamines in humans.

Montelukast Sodium (109 – 116)
(Antiasthma)

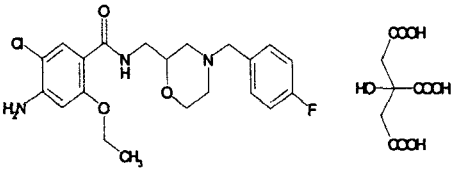
Country of Origin : US
Originator : Merck & Co
First Introduction : Finland, Mexico
Introduced by : Merck & Co
Trade Name : Singulair
CAS Registry No : 151767-02-1
Molecular Weight : 608.17



Montelukast was launched as Singulair in Mexico and Finland for the management of mild to moderate asthma inadequately controlled by inhaled corticosteroids and short-acting beta2-agonists. Montelukast can be obtained by an seven-step synthesis from 3-[2(E)-(7-chloroquinolin-2-yl)vinyl] benzaldehyde. Montelukast is a potent, selective and orally active antagonist of the CysLT1 (formerly called LTD4) receptor, thus blocking the effects of the cysteinyl leukotrienes LTC4, LTD4 and LTE4 on microvascular permeability and the activation of eosinophils. Montelukast represents the third molecule of this class which has been approved in asthma after pranlukast (1995) and zafirlukast (1996). Montelukast has been studied extensively in placebo-controlled clinical trials, in mildly or severe asthmatic patients challenged with LTD4 or exercise. A variety of acute bronchoconstricting challenges were inhibited or attenuated with all doses used. Montelukast demonstrated clinically significant improvements in the parameters of asthma control associated with an appreciable improvement in quality of life., reducing days with asthma exacerbations and allowing significant tapering of corticosteroids. Montelukast is well-tolerated and only needs to be administered once a day.

Mosapride Citrate (117 – 124)
(gastroprokinetic)

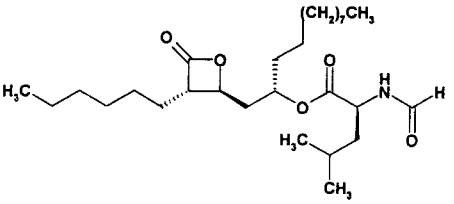
Country of Origin : Japan
Originator : Dainippon
First Introduction : Japan
Introduced by : Dainippon
Trade Name : Gasmotin
CAS Registry No : 112885-41-3
Molecular Weight : 614.05



Mosapride citrate, a new gastroprokinetic benzamide was introduced in Japan as Gasmotin for the relief of gastrointestinal symptoms in patients with chronic gastritis, gastro-oesophageal reflux, dyspepsia and post-surgery use. Mosapride is synthesized by final amidation between the corresponding benzoic acid, itself obtained in 3 steps from methyl 4-acetamido salicylate, and the appropriate 2-aminomethyl-morpholine. Mosapride is a 5-HT4 partial agonist without notable activity for D2, 5-HT1, 5-HT2, alpha-1 and alpha-2 receptors. In dogs, mosapride was as potent as cisapride to stimulate antral and duodenal motility. In most of species including man, mosapride undergoes extensive cleavage to a N-debenzylated metabolite which retains partial 5-HT4 activity but has potent 5-HT3 antagonist activity.

Orlistat (Antiobesity) (125 – 132)

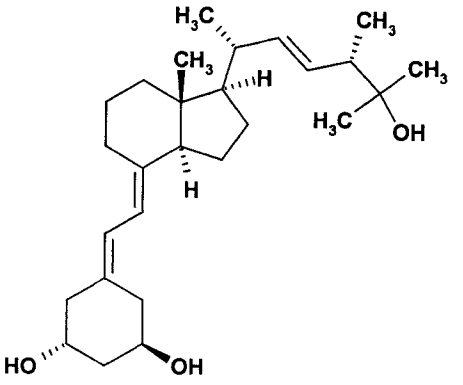
Country of Origin : Switzerland
Originator : Roche
First Introduction : New Zealand
Introduced by : Roche
Trade Name : Xenical
CAS Registry No : 096829-58-2
Molecular Weight : 495.74



Orlistat was launched in the UK as Xenical for the long-term treatment of obesity, preferably in conjunction with a moderately reduced calorie diet. Orlistat is a tetrahydro-derivative of the natural hypolipaemic lipstatin (from *Streptomyces toxytricini*) and can be obtained either by hydrogenation of lipstatine or by several different synthetic ways involving many steps from (S)-malic acid. Orlistat is a potent inhibitor of gastrointestinal lipases required for the lipolysis and digestion of dietary fat, in particular of pancreatic lipase ; as a result, it prevents the absorption of about one third of the fat contained in food and acts as an effective weight-reducing therapy. The outcomes of several clinical trials involving thousands of obese patients showed that Orlistat promotes a significant weight loss (often between 5 and 10% after one year) and improves cardiovascular risk factors such as total cholesterol, LDL/HDL ratio, blood glucose levels, insulin, blood pressure. Orlistat has minimal systemic absorption, the majority of the compound itself being recovered in the feces ; it does not affect other gastrointestinal processes, or the absorption of other macronutrients such as carbohydrates and proteins.

Paricalcitol (vitamin D) (133 – 139)

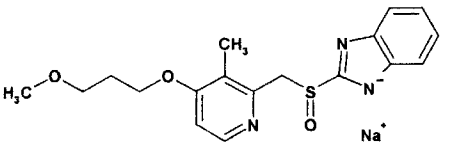
Country of Origin : US
Originator : Abbott
First Introduction : US
Introduced by : Abbott
Trade Name : Zemplar
CAS Registry No : 131918-61-1
Molecular Weight : 416.65



Paricalcitol was launched as Zemplar in the US for the prevention and treatment of secondary hyperthyroidism associated with chronic renal failure. Paricalcitol is a synthetic vitamin D2, namely a novel 1-alpha-hydroxy-19-nor-analogue in which the ring A exocyclic methylene group, typical of all vitamin D systems, has been replaced by two hydrogen atoms. Paricalcitol is the first vitamin D analogue marketed for this indication. In patients with chronic renal failure, Paricalcitol appreciably reduced levels of parathyroid hormone (PTH) without a significant difference in the incidence rate for hypercalcemia or hyperphosphatemia when compared with placebo.

Rabeprazole Sodium (Gastric antisecretory) (140 – 147)

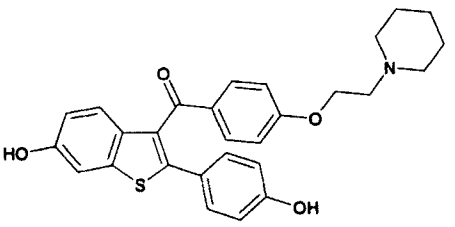
Country of Origin : Japan
Originator : Eisai
First Introduction : Japan
Introduced by : Eisai
Trade Name : Pariet
CAS Registry No : 117976-90-6
Molecular Weight : 381.42



Rabeprazole was launched as Pariet in Japan, its first market, for the treatment of peptic ulcers including gastric and duodenal ulcers. From 4-chloro-2,3-dimethylpyridine N-oxide, a six step synthesis allows access to the basic skeleton after successive condensations. Rabeprazole, a structural analog of Omeprazole, the first compound to have been marketed in this class up to now, is reported to be a more potent inhibitor of gastric H+/K+-adenosine triphosphate (ATPase) ; a common mechanism of action of this chemical class involves the conversion at low pH to a reactive sulphonamide that itself binds to cysteine residues located on the enzyme. Moreover, rabeprazole showed an antibacterial activity against Helicobacter Pylori, with a MIC90 of 1.56 µg/ml.
Rabeprazole has a faster onset of action compared with omeprazole, but a shorter duration of action, being extensively and rapidly metabolized in several animal species. In clinical studies in patients with gastric ulcers, 10 and 20 mg rabeprazole sodium once-daily significantly inhibited basal and stimulated acid output. Rabeprazole is awaiting registration in the US for treatment of gastro-oesophageal reflux disease (GORD) and other pathologic hypersecretory conditions including Zollinger-Ellison syndrome.

Raloxifene Hydrochloride (Osteoporosis) (148 – 154)

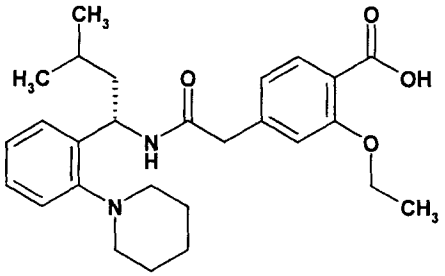
Country of Origin : US
Originator : Lilly
First Introduction : US
Introduced by : Lilly
Trade Name : Evista
CAS Registry No : 82640-04-8
Molecular Weight : 510.05



Raloxifene was launched as Evista in the US for the prevention of postmenopausal osteoporosis. It is noteworthy that this molecule was formerly under development as keoxifene for breast cancer and prostatic hypertrophy. Raloxifene can be prepared by acylation of 6-methoxy-2-(4-methoxyphenyl) benzothiophene followed by simultaneous demethylation of both methoxy groups. Raloxifen is a selective estrogen receptor modulator, exerting anti-estrogenic action on certain tissues (breast) and also estrogenic action on bone metabolism or serum lipids. In normal early postmenopausal women, 200 mg daily produced a trend towards suppression of estrogen effects. Raloxifen impeded bone loss in osteoporosis. A two-year study in postmenopausal women with an increased risk for osteoporosis showed that Raloxifen markedly prevented non-traumatic vertebral fractures. Results of several clinical studies demonstrated that Raloxifen appreciably reduced the risk of developing breast cancer. Moreover, it had favourable effect on lipid profiles without having the potential side-effects of estrogen-based therapies. Several extensions for different uses of this molecule are planned, for example growth disorder, obesity, colon tumor and skin atrophy. No serious drug-related events have been reported in the limited number of clinical trials.

Repaglinide (Antidiabetic) (155 – 163)

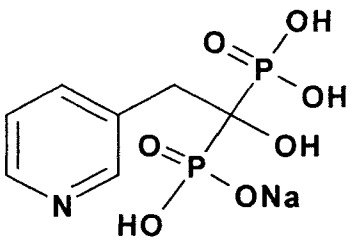
Country of Origin : Germany
Originator : Boehringer Ingelheim
First Introduction : US
Introduced by : Novo Nordisk & Schering Plough
Trade Name : Prandin
CAS Registry No : 135062-02-1
Molecular Weight : 452.59



Repaglinide was marketed in the US as an orally active hypoglycemic agent in patients with a type II diabetes mellitus, to lower blood glucose in synergistic combination with metformin, when hyperglycemia cannot be controlled by diet, exercise or metformin alone. Chiral (S)-repaglinide can be synthesized in several ways, each involving a stereoselective reduction of an imine or enamine group. Repaglinide is a nonsulfonylurea belonging to the meglitinide family, with an insulin-releasing effect mediated by pancreatic beta-cells, by closing the ATP-sensitive K+ channels that, in turn, increases the cytosolic concentration of Ca++. In several animal models, repaglinide was more efficient than glibenclamide as a dose-dependant promoter of insulin release, but its mechanism of action is probably slightly different. Repaglinide has a rapid onset of action, a short duration of action and a reduced risk of hypoglycemia compared to glyburide.

Risedronate Sodium (164 – 169)
(Osteoporosis)

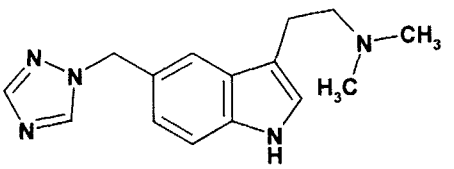
Country of Origin : US
Originator : Procter Gamble
First Introduction : US
Introduced by : Procter Gamble & HMR
Trade Name : Actonel
CAS Registry No : 115436-72-1
Molecular Weight : 305.10



Risedronate sodium was launched as Actonel in the US for treatment of Paget's disease. Risedronic acid is readily obtained by condensation of 2-(3-pyridyl)acetic acid with phosphorous acid in presence of phosphorus oxychloride. Risedronate sodium is an orally active bisphosphonate of third generation, showing more potent bone antiresorptive properties than most of prior bisphosphonates like etidronate which has been used for the same indication since 1978. The biological mechanism by which Risedronate, and generally bisphosphonates, act is not quite clear presently ; recent advances have suggested that aminobisphosphonates interfere with the HMG-CoA pathway and inhibit protein prenylation, causing apoptosis of osteoclasts and macrophages. These apoptotic effects are correlated to the anti-resorptive properties of this class of compounds. In a 2 month clinical trial involving patients with Paget's desease, 30 mg risedronate daily achieved an appreciable reduction of serum alkaline phosphatase levels, an indicator of bone stabilization, in 77% of patients, compared with 11% on etidronate at 400 mg daily for 6 months. Risedronate is also in development for the treatment and prevention of various bone diseases, particularly post-menopausal and corticosteroid-induced osteoporosis.

Rizatriptan Benzoate (170 – 177)
(Antimigraine)

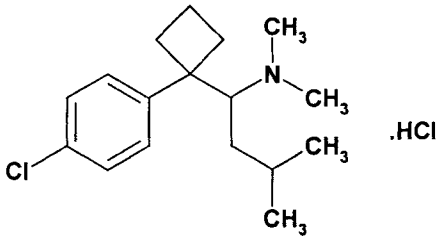
Country of Origin : US
Originator : Merck & Co
First Introduction : Mexico
Introduced by : Merck & Co
Trade Name : Maxalt
CAS Registry No : 145202-66-0
Molecular Weight : 391.47



Rizatriptan was launched for the first time as Maxalt in Mexico for the acute treatment of migraine headaches with or without aura in adults. It is the third in a new generation of triptan migraine drugs to challenge the first-generation product Sumatriptan from Glaxo Wellcome, after Zolmitriptan (Zeneca) and Naratriptan (Glaxo Wellcome).
It can be synthetically obtained by two related routes having respectively 3 and 4 steps, both starting from 1-(4-aminobenzyl)-1,2,4-triazole. Rizatriptan is a full 5-HT1D/1B receptor agonist retaining a significant affinity at 5-HT1A sites (pIC50 = 6.4), with a low affinity for non-5-HT sites. It shows craniovascular selectivity for human isolated middle meningeal over coronary arteries ; its effect on coronary artery constriction is significantly less than that for Sumatriptan. Rizatriptan inhibits the release of neuropeptides that cause swelling of cranial blood vessels. Rizatriptan has a good oral bioavailability (64% and 47% in rats and dogs respectively), and a rapid onset of action : the headache relief in

human occurs within 30 to 45 minutes of taking 20 mg. It also relieved nausea and hypersensitivity to noise (phonophobia) and light (photophobia) that are often symptoms accompanying a migraine attack. Among at least 6 metabolites identified in humans after a single oral dose (60 mg), the major urinary metabolite was the corresponding indole-3-acetic acid. Maxalt is available as a novel oral formulation of rapidly dissolving tablets or wafers that does not require liquid for ingestion.

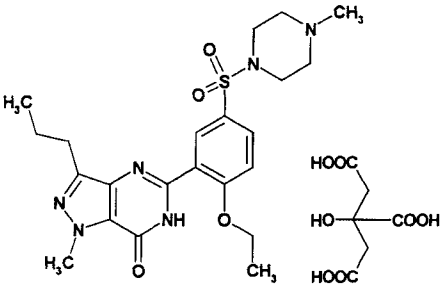
Sibutramine (Antiobesity) (178 – 186)
Country of Origin : Germany
Originator : Knoll
First Introduction : US
Introduced by : Knoll
Trade Name : Meridia, Reductil
CAS Registry No : 125494-59-9
Molecular Weight : 334.33



Sibutramine was introduced as Meridia in the US for the control of obesity, in conjunction with a reduced-calorie diet. Sibutramine can be prepared in four steps from 4-chlorobenzyl cyanide. Sibutramine acts as a serotonin and noradrenaline reuptake inhibitor ; in animal studies, it reduces energy intake by creating a satieted feeling and increases energy expenditure by enhancing thermogenesis. In a clinical study in obese patients treated with 10 or 15 mg of Sibutramine once-daily, meaningful weight loss was sustained for 12 months ; an additional study demonstrated a lack of abuse potential. An appreciable part of Sibutramine's effect would also be due to its 2N-mono and di-desmethylmetabolites, which are very potent monoamine reuptake inhibitors themselves.

Sildenafil Citrate (Male Sexual Dysfunction) ((187 – 194)

Country of Origin : UK
Originator : Pfizer
First Introduction : US
Introduced by : Pfizer
Trade Name : Viagra
CAS Registry No : 139755-83-2
Molecular Weight : 474.58

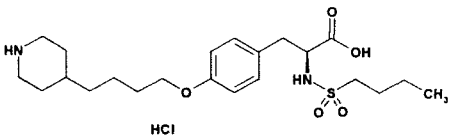


Sildenafil was launched as Viagra in the US for the treatment of organic or/and psychological male erectile dysfunction (ED). Sildenafil can be obtained by functional rearrangement of the corresponding 5-aryl 1,3-dialkyl pyrazolo[4,3-d]pyrimidin-7-one, itself synthesized in a seven-step sequence from a pyrazole-5-carboxylate. Sildenafil is a potent and selective inhibitor of type V cGMP phosphodiesterases (PDE5) ; IC50 = 3nM on isozymes from human corpus cavernosum tissue. This orally-active therapy is completely new and presents advantages over the classically recommended options such as vacuum

constriction devices, drug injection or prosthesis implantation ; for these reasons, this first drug is likely to have a large acceptance fot the treatment of male ED. In experiments with incubated rabbit penile tissue, Sildenafil was shown to cause accumulation of cGMP. By inhibiting the enzyme PDE5, the predominant isozyme in the corpus cavernosum, Sildenafil induces an elevation of levels of second messager cGMP, which is involved in the regulation of vascular tone ; it was suggested that the specific elevation of cGMP due to Sildefanil would mediate an enhancement of nitric oxide-dependent relaxation of corpus cavernosal tissue. Several clinical studies using 10-100 mg Sildenafil have confirmed a good effectiveness and tolerability in healthy males. New trials in women with sexual disfunction have been initiated and positive results could enlarge the potential market of this drug.

Tirofiban Hydrochloride (195 – 202)
(Antithrombotic)

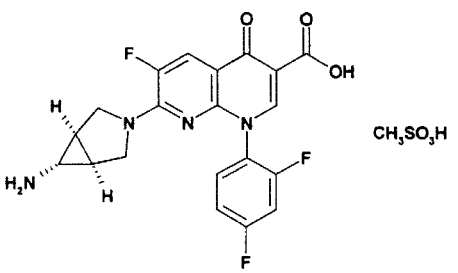
Country of Origin : US
Originator : Merck & Co
First Introduction : Switzerland and
the US
Introduced by : Merck & Co
Trade Name : Aggrastat
CAS Registry No : 142373-60-2
Molecular Weight : 477.06



Tirofiban was launched as Aggrastat in Switzerland and the US for patients with unstable angina or non-Q-wave myocardial infarction to prevent cardiac ischemic events. Tirofiban is synthesized from (S)-tyrosine by a four-step process. Tirofiban is a novel highly potent antiplatelet agent that inhibits the interaction of fibrinogen with GPIIb/IIIa, an activated platelet membrane-bound glycoprotein complex. It prevents arterial thrombus formation in a dose-dependent manner. Clinical efficacy was highly significant with an absolute and relative risk reduction. In these studies, treatment with tirofiban was well tolerated, without significant adverse effects.

Trovafloxacin Mesylate ((203 – 210)
(Antibiotic)

Country of Origin : US
Originator : Pfizer
First Introduction : Switzerland and the US
Introduced by : Pfizer
Trade Name : Trovan
CAS Registry No : 157605-25-9
Molecular Weight : 512.47



Trovafloxacine mesylate, a new fluoroquinolone, was launched in Switzerland and the US, in both oral (Trovan) and iv (alatrofloxacin, N-Ala-Ala substituted prodrug of trovafloxacin) formulations for adult use as broad-

spectrum antibiotic agent in diverse acute bacterial infections, particularly community-acquired respiratory infections (ie nocosomial pneumonia). In a clinical study of acute exacerbations of chronic bronchitis, trovafloxacin at 100 or 200 mg daily demonstrated advantages over amoxicillin, cephalosporins and clarithromycin. A favorable pharmacokinetic profile allows only once a day dosing.

References

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Chapter 32. Pharmacogenomics and its Impact on Drug Design and Optimisation

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Introduction - Genomics is defining all protein targets available for drug discovery in the human, while chemoinformatics, reviewed in Annual Reports last year (1), is providing an architecture in which to formulate corresponding chemical diversity information. The developing interface between these two areas - pharmacogenomics - promises to provide a global platform through which to unify the study of pharmacology at both the cellular and molecular level.

In current usage, the term pharmacogenomics includes both the use of genomics to study a drug's biological effects, as well as the study of genetic variation in drug response observed in patients, although traditionally the latter has been referred to as pharmacogenetics (2). A comprehensive pharmacogenomics platform has several elements, some of which are illustrated in Figure 1 below.

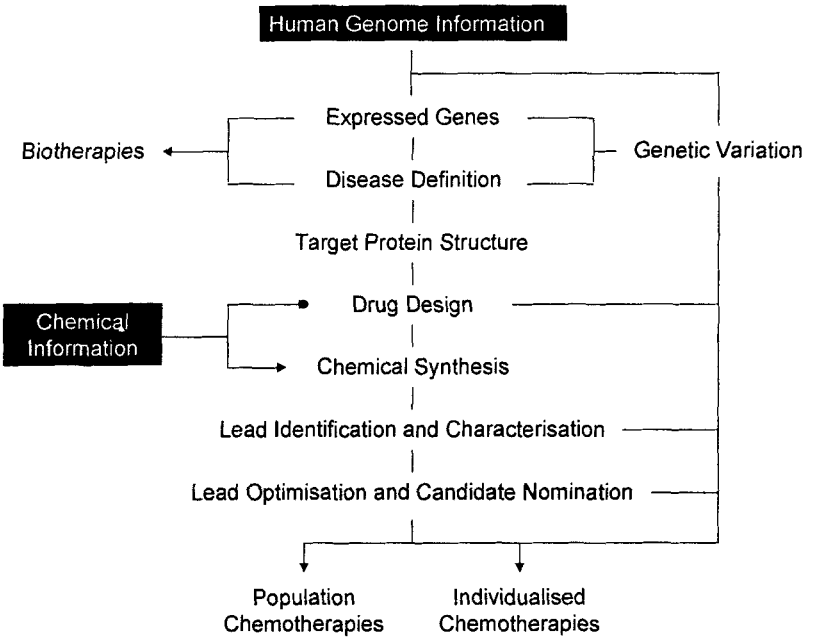

Figure 1 Pharmacogenomics uses information from both genomics and chemoinformatics, to provide a platform which addresses pharmacology from population to individual patient levels

The linkage between the genetic basis of drug response and the direct observation of the effects of a compound on biological systems at a molecular level, reflects the convergence of two distinct themes in genomics, one focussing on DNA's structure, the other on its dynamic function, effected through the processes of cellular RNA and protein synthesis. The breadth of the latter read-outs, reflecting contributions from the entire genome and proteome, places drug action on a broad biological footing.

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The development of technologies for observing the changing patterns of gene and protein expression characteristic of particular biological systems now permits 'genome-wide' pharmacology to be carried out on drugs as they act on specific cell and tissue systems, *in vitro* and *in vivo*. Spanning biological and chemical perspectives, pharmacogenomics thus provides an integrated view of processes such as drug metabolism, pharmacokinetics and toxicology, complementing the molecular pharmacology approaches more typical of traditional drug discovery.

Expansion of gene information - The force which is propelling this revolution in the way we look at drug action is the 'Human Genome Project', based on public domain gene discovery efforts as well as several well-established proprietary programmes. Together, these programmes have begun to define the human genome's wealth of feasible drug discovery targets (3).

There are two main strategies for defining a complete set of expressed human genes: one relies on characterising expressed mRNA sequences, the other uses computational methods to extract coding sequences from chromosomal DNA sequences (4). The convergence of information from these two approaches will raise our knowledge of the working human genome, and the drug targets it contains, to the same levels of detail as that of model organisms such as yeast and *C.elegans*, two eucaryotes whose genomes have now been completely sequenced (5,6).

With access to gene sequence has come the development of new bioinformatics approaches to classify genes. Increasingly sophisticated multiple sequence alignment and protein homology modelling approaches have led to the definition of specific structural 'motifs' through which to identify and classify new members of related gene families. A recent summary and extensive review of new 'knowledge bases' in this area have recently been published (7,8).

Sophisticated gene function analyses, in both model and human systems, are now being undertaken (see for example the work on *C.elegans* described in ref (9)). An interesting approach to genome-wide functional analysis has been described in which individual human cells can be genetically 'tagged' and then screened to search for particular functional pathways (10). Such studies serve to identify new points of intervention in the biochemical pathways underlying disease, especially when linked to techniques of gene 'knock-out' in animals. An impressive example of the insight provided by gene knock-out techniques has been the recent confirmation of Protein Tyrosine Phosphatase 1B as a potential target for drug therapy in type 2 diabetes and obesity (11,12). A link between these two diseases had long been suspected, but the demonstration that increased insulin sensitivity is accompanied by obesity resistance in these animals is an elegant demonstration of the power of such technologies to define physiological processes at the genetic level, while also demonstrating their economy.

Of particular interest to this review is progress which has been made in linking human disease to genetics. The past year has seen the announcements of both public and proprietary programmes to provide a 'first draft' of the entire sequence of the human genome by the end of 2001 and construct definitive 300,000-marker genetic maps (13,14). These efforts will lay the foundations for the identification of functional polymorphisms within therapeutic targets and human drug metabolising enzymes, a key step towards understanding how genetic diversity within patient populations can affect both the efficacy and safety of drug therapy, a major aspiration of pharmacogenomics (15).

Using gene expression measurement to define biology - Biological processes can now be dissected into molecular pathways acting at the cell and tissue level using genome-wide expression analysis. Several reviews of this technology have appeared during

the past year (16,17), and a number of cell-based systems have been studied in depth, including fibroblasts (18), haematopoietic cells (19) and lymphocytes (20). To collate and expedite the interpretation of such gene expression data, a number of laboratories have begun to construct data warehouses to store, and powerful software to analyse this data (19,21,22). Of particular importance to pharmacologists and toxicologists will be the development of methods to link drug effects with particular pathways of efficacy and/or toxicity. The commercial availability of gene matrices from a number of sources promises to provide robust and reliable platforms for such gene expression analyses (23-25).

Using gene expression as a surrogate marker of drug action - The use of gene and protein expression end-points as surrogate markers of pharmacological action has been well-documented. From the measurement of p24 levels during HIV infection, to the induction of adhesion proteins during macrophage activation, single molecules or small 'families' of molecular markers have been widely used as a basis for monitoring biological processes and establishing screens for drug discovery. The exciting new prospect is the use of genome-wide RNA and protein profiling approaches to examine the entire catalogue of transcriptional events involved in generating such dynamic biological responses, not only their isolated end-points.

Using a panel of 60 tumor cell lines, workers at the NCI have begun to link responsiveness to compounds to measurements of changing phenotype, including gene expression, with the aim of exploiting key genes as therapeutic targets or treatment diagnostics (for an initial discussion of this approach see ref (26)). Similar studies defining the mitotic phenotype and exploiting combinatorial chemistry to directly target individual protein kinases have also appeared during the last year (27,28).

A recent review of chemokines as proinflammatory mediators and the identification of non-peptide chemokine receptor antagonists nicely illustrates the opportunities and challenges for focussed drug discovery presented by complex biological processes such as inflammation (29). The inflammatory process involves many specialised cell types, each contributing to the overall inflammatory process. However, with in-depth cDNA sequencing to define the majority of the 'players' at the molecular level, coupled to mRNA profiling to evaluate the dynamic relationships between genes induced during the cellular activation process (ref (20) and Figure 2 below), valuable information on the pathways involved in the biological response and the proteins which comprise them can be obtained.

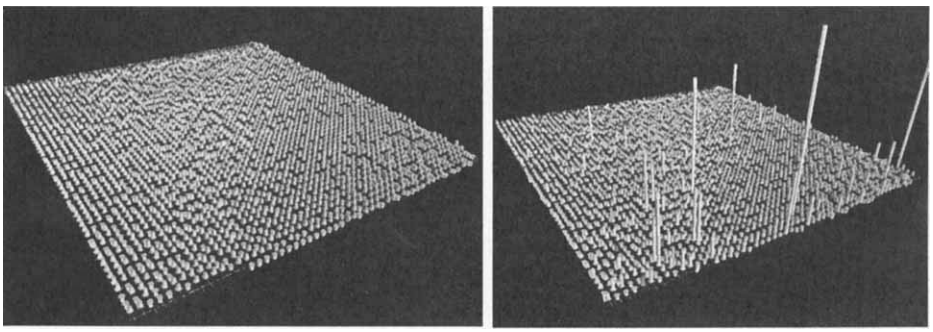

Figure 2 Gene expression changes amongst 3,000 selected human genes induced in human THP-1 cells treated with the phorbol ester PMA, using a microarray (23).

With defined biological systems in hand and well-characterised molecular markers for examining the effector pathways expressed within them, it should be possible to establish, quickly and relatively easily, a focussed set of expression-based screens to

identify and characterise NCEs and biotherapeutics with precisely-targeted biological activities. The applications of these approaches to drug design and lead optimisation are discussed below.

Drugs which target gene expression - Agents which directly activate or repress gene expression are prime candidates for discovery through approaches based on transcriptional analysis. This is a complex and rapidly-developing field (30), but one interesting example of current importance is the case of peroxisome proliferator activated receptor agonists (PPAR agonists).

PPAR α, δ and γ are related proteins which form heterodimers with retinoic acid receptors which upon ligand-binding transactivate the promoters of specific genes sharing the PPAR-binding DNA motif (31). Amongst these are genes which control lipid metabolism, such as lipoprotein lipase and acetyl CoA synthetase. PPAR ligands sensitize cells to insulin, thereby providing a potential therapy for Type 2 diabetes. Thiazolidinediones such as rosiglitazone show selectivity for PPARγ. Fibrates such as clofibrate act on PPARα (32). Some structures are shown in Figure 3.

A crystal structure of PPARγ and rosiglitazone has been produced (33,34). The crystal structure shows that the thiazolidinedione (TZD) head group binds into a T-shaped pocket within the receptor. The carbonyl groups of the TZD form hydrogen bonds with two histidine residues and a lysine. Alpha-substituted carboxylic acids can replace the TZD head group. The end distal from the TZD is free to bind non-specifically in a large cavity.

A very large study on structure activity relationships of N-(2-benzoylphenyl)-L-tyrosine analogues has been performed by GlaxoWellcome scientists who report the molecule named compound 18, containing elements from both fenofibrate and rosiglitazone, to bind with high affinity to PPARγ and with low affinity to PPARα (35-37). The strong transactivation observed via PPARγ but not via PPARα, would make it an interesting molecule to study by gene expression analysis, side by side with other replacements showing selectivity between these two transactivators, such as those reported by Merck (38).

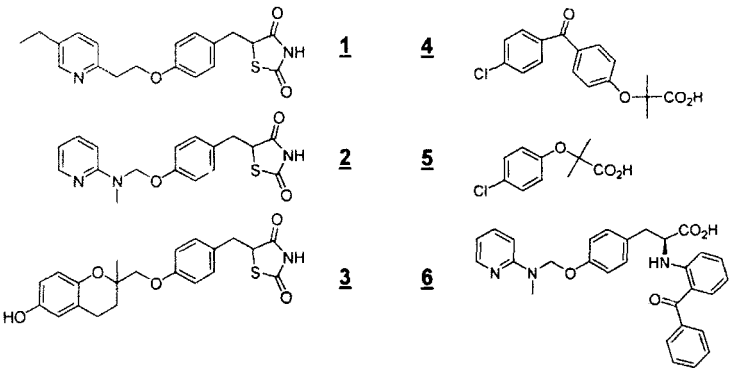

Figure 3 Shown on the left are thiazolidinedione compounds pioglitazone (1), rosiglitazone (2) and troglitazone (3), which activate PPARγ; on the right are two fibrates, fenofibrate (4) and clofibrate (5), which activate PPARα, with Compound 18 (6), a hybrid molecule with moieties from fenofibrate and rosiglitazone, which activates PPARγ.

Gene expression for lead optimisation - Toxicology is a particularly exciting area in which to apply pharmacogenomics. Many chemical tools are available through which

to correlate well-characterised histopathologies to gene expression patterns, both temporally and spatially. The possibility of directly linking (ultra)structural biology to gene expression and proteomic patterns, both *in vivo* and *in vitro*, to produce a predictive database for compound screening is very attractive (39). The sensitivity of gene expression profiling techniques exceeds that of histopathology, and has a much higher informational content. The challenge will be the interpretation of the resulting information in drug safety terms.

The utility of gene expression analyses in toxicology is clearly illustrated by the PPAR agonists, an important drug class carrying some suggestion of mechanism-based hepatotoxicity. The key questions to resolve for PPAR approaches are: 1) the role of PPARα, PPARδ and PPARγ in the physiological control of basic biological processes such as lipid metabolism and mitogenesis, especially in the liver; 2) the mechanisms by which fibrates and TZDs, as drug classes, elicit any hepatic pathology; and 3) species differences in the development of any such hepatic pathologies. PPARα has been shown to regulate expression of human aspartate and alanine amino transferases, as well as hepatic gluconeogenesis (40). Although these transferases are toxicological markers of liver tissue damage, their induction appears to be part of the normal transcription programme. Peroxisome proliferators have also been shown to induce mitogenesis in hepatocytes in the absence of exogenous growth factors (41). Glucocorticoids are required for this mitogenicity and it has been proposed that the mechanism for this is via induction of PPARα. Although, there is no evidence of an increased incidence of cancer in man with the clinically available fibrates, an objective study of their action in both isolated hepatocytes and in liver *in vivo* using gene expression techniques could clarify the mechanistic basis of their effects. A recent study suggesting alterations in PPAR consensus binding sites in PPAR-binding promoters in patients as a distinguishing feature between rodents and humans would support doing these studies (42).

Can pharmacogenomics also assist in drug design? - The 'holy grail' of drug discovery is the *in silico* design of drug candidates. If this were possible on a routine basis, then the NCE discovery rate might actually rise in parallel with our increasing understanding of the molecular basis of disease. As we have seen, the Human Genome Project is now producing sequences to cover all genes relevant to human disease, including therapeutic targets. What do we need to put into place to capture this value in new drug structures?

Databases for macromolecular structure - *De novo* drug design is hampered by the need for an acceptable 3D structure of the target molecule. However, the rate at which new crystallographic determinations of protein structures are being published lags dramatically behind the rate of sequence discovery from genomic data. Table 1 illustrates the small numbers of existing new macromolecular structure determinations. In 1998 less than 1000 good quality determinations (<2.5A resolution) were produced.

Table 1 Brookhaven Protein Data Bank entries for 1998

Technique	Last Year (1 Jan 1998-31 Dec 1998)	Cumulative (1 Jan 1972-31 Dec 1998)
X-ray diffraction	1285	8105
Resolution 1.5-2.5A	974	6143
NMR	269	1525
Models	217	217

As if throughput of target structures were not enough, crystal structure itself is a single snapshot of macromolecular structure in a restricted environment. Where

solution NMR determinations can be compared with X-ray structures, considerable conformational variation in structure becomes evident (Figure 4). The acknowledgement of target site plasticity raises a fundamental question. How valid is drug design closely tailored to a single conformation of the site? Hitherto, most *de novo* design algorithms, have been based on a single fixed structure for the site. If this constraint should be lifted, perhaps numerous models of the site should be used instead. This raises the question of whether homology models can provide site data that is as good as the variation in the NMR data.

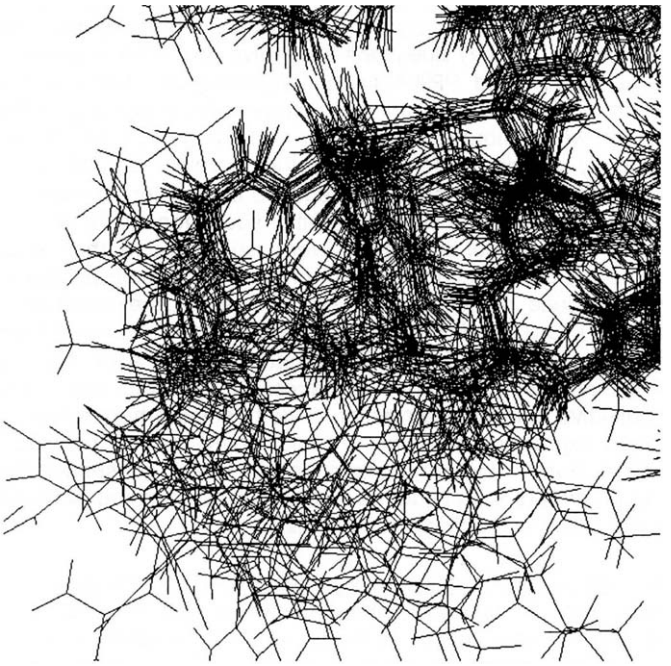

Figure 4 A segment of a mutant insulin protein (PDB refcode 1A7F) showing 20 NMR structures with very large conformational variation particularly in the loop region at the bottom of the picture.

A landmark series of papers on protein structure determination, classification and usage appeared in Acta Crystallographica series D in 1998 (43-48). Protein modelling from sequence data is founded on protein sequence alignment techniques. There are three basic components to the alignment problem: how to score the alignment, how to insert gaps between non-corresponding elements of structure, and how to optimise the alignments. The scoring system BLOSUM has recently been tested extensively and shown to be better than competing scoring systems. An optimal alignment procedure maximises the score but at the same time minimises the gaps. A variety of multiple sequence alignment techniques is given by Barton (43). The structural classification of proteins continues to be developed in the SCOP database. The classification deals with the hierarchy of class of the core e.g. α, β, α/β, $\alpha+\beta$, the fold, the superfamily, family and domain. The value of the database lies in its ability for the user to make structural comparisons between proteins. It is particularly useful for examining drug-target similarities (44). An essentially similar database, CATH, contains the class, architecture, topology and homologous family relationships (45). Other databases of protein architectures are continually evolving (46,47). Of direct interest to drug design, has been the development of a database of protein-ligand complexes, ReLiBase (48). This database has been developed with a variety of structural analysis and query tools that can be linked to it thus simplifying the analyses of structural complexes.

Homology Modelling - X-ray crystallography is a slow process compared with gene sequencing. The only way that sequence data can be utilised rapidly by the pharmaceutical industry is by large scale homology modelling. Sanchez & Sali in an important paper, have attempted to create structure models from the yeast genome (49). They used 1071 sequences and employed ALIGN and MODELLER to create homology models automatically. This procedure has provided clues to the function of the proteins by identifying the folds and 3D motifs known to bind to specific ligands e.g. the identification of SH3 domains. Steady progress is being made. 3D-computational models for half the genome proteins may be available by 2003 (50).

Features of Sites Relevant to Drug Design - In general terms drug binding to specific sites is reversible and achieved through non-covalent interactions such as hydrogen bonding between site and ligand, hydrophobic interactions, electrostatic interactions, steric interactions and those mediated by water molecules in the site. However, if the site is an enzyme there may be more specific interactions such as those between the ligand and metal ions in the site, or interactions between cations and the π clouds of aromatic rings. These features of drug-site interactions have been incorporated into scoring functions for *de novo* design. The scoring function enables a designer to prioritise the output from an automated *de novo* generation algorithm. The LUDI programme formerly used a function SCORE 1 to calculate the predicted binding constants for protein-ligand interactions. A new approach, SCORE 2, has augmented the scoring function by improving the representation of polar contributions and aromatic interactions (51). This function was tested on a set of 82 protein-ligand interactions; it gave a standard deviation between experimental and predicted binding constants of 7.3kJ/mol. As a caveat to this approach, large-scale testing of a different scoring function showed that the predicted and experimentally found affinities within a series of synthesised molecules showed a large variation, predictably where the small molecule structures were not represented in the training set (52).

Small Molecule Similarity and Diversity - Molecular similarity procedures are now reasonably mature, both in terms of defining similarity between molecules in a set and in the utility of the similarity concept to search for like molecules in a database of compound structures (53). Another application of similarity work is to the development of virtual chemical libraries before synthesis. There is a concept in lead optimization of neighbourhood. Molecules close to a hit or lead compound need to be synthesised to explore the similarity space, and by implication molecular interaction space between the lead and its binding site even though the structure of the site may be unknown (54). The neighbourhood procedure is applied to a small selection of hits and the descriptors of the activity are determined. Molecules containing these descriptors are searched for from a virtual database within a defined neighbourhood radius and where possible these are synthesised in a combinatorial format.

Molecular diversity within virtual libraries is also important for drug design; a judicious selection of representatives enables the library to cover a large amount of diversity space with a small set of chemical reactions (55,56). These molecular libraries can be built with "drug-like" skeletons from a selection of representatives obtained by optimizing the dissimilarity. Another new development in combichem design methods - RECAP - a retrosynthetic combinatorial analysis procedure (57) takes the Derwent World Drug Index and fragments it; firstly so that fragments can be assembled by combichem at a future date and, secondly so that structural building blocks can be identified which are correlated with therapeutic effects. The fragmentation is performed along eleven bond cleavage types and structural motifs are correlated with therapeutic activity. Thus it should be possible to build combichem libraries around these motifs to search for new lead compounds with distinct biological effects.

Pharmacogenomics complements drug design in the discovery process - In a previous section we saw how gene expression analysis could provide a rapid and efficient approach to drug profiling. The approach gains real power, however, when a 'snapshot' of a compound's biology is needed - exactly the situation arising when a number of drug designs have to be assessed for efficacy or toxicity. Although these approaches are in their infancy, the resolving power of the technology is certainly capable of identifying 'actives' from a set of disparate structures, given appropriate biological end-points. Simultaneous identification of specific toxicity 'signatures' in the same structures would be an added bonus.

Genetic variability in targets and drug metabolism pathways - A new challenge for drug designers and medicinal chemists alike lies in exploiting, or avoiding, the genetic variation evident in drug targets and drug metabolism enzymes (2,58). Pharmacogenomics has been divided into pharmacodynamic and pharmacokinetic areas, representing structural variation in pharmacological targets and drug disposition and metabolism proteins, respectively. Retrospective population studies focussed on the pharmacogenetics of drug response are currently underway and pharmacogenetic considerations are beginning to feature in the screening and optimisation processes for drug candidates (15).

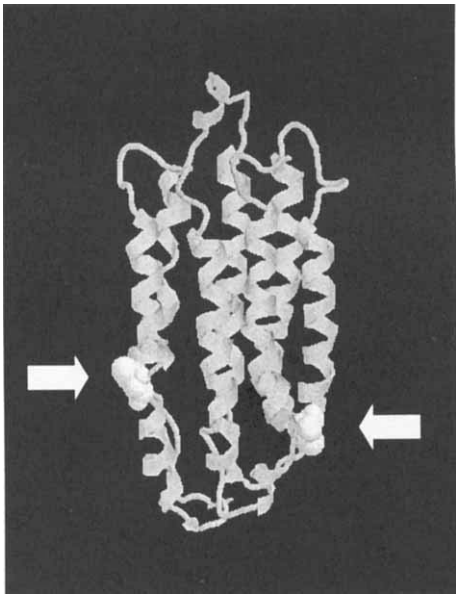

Figure 4 The sites of two mutations in the human β2-adrenergic receptor observed in clinical populations, on the left Thr164, on the right Val34 (59).

A particularly well-characterised example of 'pharmacodynamic' pharmacogenomics is genetic variation in the β2-adrenergic receptor, variously implicated in heart failure, asthma and obesity, as well as variable response to β2-adrenergic receptor agonists (reviewed in ref (59)). Figure 4 shows the positions of two of the four published receptor mutations superposed on the 3-dimensional structure of the receptor. Thr164 adjoins the Ser165 which itself flanks the amine binding site of the receptor. It seems reasonable therefore that mutation at Thr164 should compromise receptor function. As information accumulates on the location of such mutations within proteins, it may prove possible to associate 3-dimensional structure alteration with altered function, both at the pharmacological and biochemical levels, leading to prediction of the resulting biochemical effects *in silico.*

Single nucleotide polymorphisms - The assembly of a single nucleotide polymorphism (SNP) map for the genome, representing at the same time a set of characterised markers, or landmarks, spread throughout the genome, and individual variations within particular genes, some of which will undoubtedly be associated with disease, promises to throw light on the genetic variability inherent in human populations, in itself an important biological question (60,61). Such information promises to have fundamental effects on the way in which drugs are prescribed (15, 62). The prospect of accomodating all possible variants in drug targets within a particular drug discovery campaign seems less realistic - unless we can precisely define the structural and pharmacological variation they produce without first having to clone, express and crystallise all the corresponding proteins.

Conclusions - Pharmacogenomics provides an operational link between medicinal chemistry and biology, and enables structure-activity relationships between drug candidates to be determined on an objective and informed basis. The implications for both drug discovery and drug development, especially toxicology, are clear: the provision of detailed information on the way in which drugs work within a biological setting can only enhance the productivity of the industry. A particularly interesting aspect of progress in this area is the relationship between pharmacogenomics and *de novo* drug design, where rapid, detailed feedback on the biological properties of novel structures is much in demand.

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Chapter 33. Malaria: A Third World Disease in Need of First World Drug Development

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Communicable diseases account for approximately one third of all of the deaths in the world according to the 1999 World Health Report [1]and new drugs and vaccines are urgently needed both for prevention and treatment of infectious diseases. Antimicrobial drug resistance has reduced the efficacy of existing drugs and for many diseases effective vaccines are not available. Major pharmaceutical companies have developed renewed efforts in the area of infectious diseases, focusing on modern approaches including the use of genomics in target discovery and validation, combinatorial chemistry and high throughput screening methodology for lead discovery and structure based drug design. There is great promise that genomics approaches will identify novel drug targets, not found by conventional strategies and that we will enter a new period where novel drugs are developed for these new targets. However, many of the infectious diseases which lead to the over 50 million deaths per year are diseases which primarily affect those living in countries with few economic resources and no pharmaceutical industry and as a world community we must address the challenge of how we can apply the truly exciting developments of the modern pharmaceutical industry to these diseases where there is great need[2]. This piece focuses on malaria as an example - the Plasmodium falciparum genome is being sequenced and we are beginning to understand some fundamental features of the pathogenesis of the disease. The challenge facing the research community is to bring this knowledge to the treatment and prevention of this disease. Many other diseases such as tuberculosis present similar challenges to the research and world health community and we must find a new paradigm in a skills based collaboration between the public/academic sector and the pharmaceutical/biotechnology industry to meet this challenge.

MALARIA: THE BURDEN OF DISEASE

Malaria remains a major health threat in the world today as it has for the last century, a major cause of death, particularly among children and women, primarily in Africa. According to the most recent World Health Report[1], there are nearly 300 million new cases of malaria each year and over a million children die each year, most of them before the age of 4 years old. The economic and social burden of malaria infection and disease is also very large with estimates of nearly 40 million disability adjusted life years (DALYs, a measure of disease burden used by WHO) attributable to malaria infection. While a world health problem, the geographic distribution of the disease is not uniform throughout the world. Approximately 90 % of both the morbidity and mortality due to malaria occur in Africa and the Eastern Mediterranean and the remainder primarily in South East Asia, the Western Pacific and Central and South America. Malaria is directly responsible for one in five childhood deaths in Africa and indirectly contributes to death from the other major childhood diseases including

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respiratory infections and diarrhea disease. Today, in the United States and Western Europe, most of the malaria infections are imported by travelers and with the increasing globalization of commerce, the number of travelers to areas of high risk in increasing each year.

DRUG RESISTANCE IS SPREADING

The rapid spread of resistance to antimalarial drugs presents a major threat[3]. The safest and least expensive antimalarial drugs are rapidly losing their effectiveness and there are few drugs to replace them. Resistance to new antimalarials has developed very rapidly, in part due to the fact that many drugs are closely related or have the same target enzyme or biological process. For example, resistance to chloroquine developed only after the drug had been in extensive use for 20 to 30 years, while resistance to mefloquine, a related compound developed in less than four years. This observation, namely the rapid development of resistant organisms is not unique to malaria but is seen for many infectious diseases, including both viral and bacterial diseases. In many diseases, multi-drug therapy has been introduced to help reduce the emergence of resistant organisms. A similar approach has been used in malaria treatment. For example, in Thailand, mefloquine was originally combined with the antimalarial drug Fansidar itself a combination of pyrimethamine and sulfadoxine, targeting two steps in the folate metabolic pathway. This strategy may have delayed the emergence of resistance; however, resistance to both mefloquine and Fansidar emerged rapidly in Thailand. A similar scenario of relatively rapid emergence of resistant organisms has been observed for all of the antimalarial drugs in use today. Thus, we are rapidly approaching a time when there will be multidrug resistant Plasmodium falciparum and no effective drugs to treat the disease.

NEED FOR NEW DRUGS

There is an urgent need for new, effective and inexpensive drugs to treat malaria, however, there are no new drugs in the pipeline. Yet, new drug development for malaria is confounded by the economic reality of the disease distribution. As mentioned above, malaria is a disease primarily of Africa, Asia and South America, areas with limited economic resources and without a developed pharmaceutical industry. Major pharmaceutical companies have in the past had active drug development programs for malaria, however over the past decade almost all of these efforts have been abandoned. In a survey of the major pharmaceutical companies done by the Harvard Malaria Initiative in1997 and 1998, only two had any activity in malaria and this was primarily focused on late stage development of compounds discovered more than a decade ago[4, 5]. None reported either current or planned discovery and development efforts. The reason for these changes are multifactorial, however, the size of the market for antimalarial drugs estimated to range from $100 to $200 million per annum is likely to be a contributing factor. Thus, the world health community faces a dilemma, there is an urgent need for new drug discovery for malaria, yet the traditional source of new antimalarials, namely the major pharmaceutical industry has disappeared.

A new paradigm for antimalarial drug development is needed. One potential approach is through public sector funding; however, research on malaria remains severely underfunded. According to a 1996 report released by the Unit for Policy Research in Science and Medicine (PRISM) of the Wellcome Trust, a private charity

that is one of the major sponsors of biomedical research, expenditures for malaria research equal approximately $42 per death, while expenditures for research on diseases like AIDS, cancer, or asthma are 100 to 1,000 times higher. According to the PRISM report, in 1993 only $84 million was spent on malaria research worldwide. The largest share—more than one quarter—was devoted solely to vaccine development. Indeed, for decades, the hope of defeating malaria has rested largely on the belief that a vaccine for the disease is just around the corner. Monies spent from public sector funding (governmental or international agencies and private foundations) on drug discovery and development represented less than 5% of the total funds or an amount less than most drug companies spend annually on any single discovery/development project. There are some indications that additional resources for malaria drug development will be available with the next few years. Recent efforts led by the WHO Roll Back Malaria Initiative has established the Medicines for Malaria Venture, which seeks to promote public/private collaboration through joint funding of promising new antimalarial drug development projects. The Plasmodium falciparum Genome Project Initiative will provide an excellent opportunity for rapid new target discovery research and will also promote more initial efforts in drug discovery. The Multilateral Initiative on Malaria (MIM) has increased awareness of the need for more malaria research has led to new funding initiatives by several agencies.

MALARIA: THE BIOLOGY AND DRUG TARGETS

An understanding of the biology Plasmodium falciparum and the pathogenesis of the disease provides many opportunities to identify unique targets for intervention and this fundamental work is underway in many laboratories and field sites around the world. Included here is a summary of some of the ongoing work using the parasite life cycle as a starting point.

Malaria infection is initiated by the bite of an infected Anopheline mosquito[6]. In humans, four species, Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale cause disease. P. falciparum and P. vivax are the most common infections and interestingly, drug resistance is primarily manifest in Plasmodium falciparum infections, although recent evidence suggests that resistance is emerging in P. vivax as well. Infective forms of the parasite named sporozoites are carried into the human host in the mosquito saliva and rapidly are carried to the liver where they invade liver hepatocytes via a receptor ligand mediated adhesion of the parasite. The parasites establish infection in the liver hepatocytes and over a period of ten days in P. falciaprum multiply from a single parasite to 10,000 parasites, with one of the fastest rates of DNA replication known in eukaryotic cells. Both the invasion of the sporozoite into the liver cell and the replication within the liver cell are potential targets for intervention and drugs such as Fansidar and proguanil which target DNA synthesis are effective as prophylactic drugs, presumably via targeting DNA synthesis in the liver stage.

Upon maturation to merozoites, the parasites are released from the liver and invade human erythrocytes, again via a receptor mediated process[7]. The merozoite invades the red cell generating a vacuolar membrane containing both host and parasite derived lipids and proteins. Once inside the infected red cell, the parasite begins its replication cycle - over a 48-hour period, the parasite multiplies from a single merozoite to 20 new daughter merozoites, each of which is capable of invading a new red cell. Within a few days in a non-immune host, the number of parasites can reach 1% or 10^{11} parasites. All of the symptoms including fever, anaemia, cerebral

malaria, and the morbidity and mortality of the disease are associated with the erythrocytic stage of the infection and the majority of antimalarial drugs are directed at this stage in the parasite life cycle[8, 9].

One important target for antimalarial drugs has been the process of hemoglobin digestion, which appears to be essential for parasite growth[10]. The parasite uses the red cell cytoplastic hemoglobin as a source of amino acids and digests during the course of the 48-hour replicative cycle over 90% of the hemoglobin in a red cell. The heme moiety is a toxic byproduct and is normally polymerized into an inert crystalline substance, -hematin. Chloroquine and related amino quinolines inhibit this polymerization[11]. Further, there are specific aspartyl and cysteine proteases in the food vacuole, where hemoglobin digestion occurs and treatment of parasites with protease inhibitors specific for these enzyme results in parasite killing[10].

Plasmodium falciparum has evolved multiple mechanisms to evade the host immune response including the relatively clever strategy of sequestration. This sequestration is a contributory factor in cerebral malaria because bound parasitized red cells block small capillaries and initiate a cascade of events including stimulation of TNF which rapidly leads to coma and often death of the patient. Prevention of sequestration may be an excellent target to prevent cerebral malaria, the major cause of death in children. The parasite expresses a series of proteins on its surface which mediate binding of the parasitized erythrocytes to capillary walls via mammalian cell ligands such as CD-36, ICAM 1 and chondroitin sulfate[12]. A large family of var genes encode these surface receptors which share binding domains for the ligands, but are otherwise diverse in sequence. Sequential expression of different var genes leads to another immune evasion mechanism that of antigenic variation.

To complete the cycle, a subset of the parasites in infected red cells follow a different developmental pathway and form gametocytes. In P. falciparum, this develop takes approximately 10 days and several drugs are known to inhibit this development including Fansidar and the artemisinin derivatives. Once taken up by a mosquito in a blood meal, gametes are released and fertilization occurs in the mosquito midgut. The zygote parasite differentiates into a motile form and migrates across the gut wall where it develops into an oocyst, undergoing reduction division and then replication to produce approximately 10,000 sporozoites from a single zygote. It is during this portion of the life cycle that genetic exchange can occur between different parasites. The obligate sexual exchange which occurs at each transmission cycle is thought to play a major role in the rapid spread of traits such as drug resistance through parasite populations and thus strategies to reduce transmission either through a vaccine or transmission blocking drugs may have a significant effect on the spread of drug resistance[7, 13]

THE CHALLENGE OF DRUG RESISTANCE

Over the past three decades, the problem of drug resistance in the Plasmodium falciparum has been increasing. It has spread from limited geographic foci to the current global distribution such that in 1998 the majority of the Plasmodium falciparum infection show reduced susceptibility to at least one drug. Furthermore, the parasites have become resistant to multiple drugs. As each new drug is introduced into widespread clinical and field use, resistance has appeared, often within a few years. Thus, in many areas where P. falciparum is endemic, parasites are resistant to multiple drugs. This presents an acute clinical problem, namely the severe limitation of effective drugs in many malaria endemic areas. Drug resistance is not unique to

malaria but is a problem in the treatment of most microbial infections and many neoplasias. The problem is more acute for malaria because of the limited number of antimalarial drugs, although the situation with certain bacterial infections is rapidly converging with that of malaria, namely the emergence of resistance to all available drugs. Antibiotic resistance represents a major threat to public health both in the United States and throughout the world and new approaches and paradigms will be needed to address this increasingly serious problem.

STRATEGIES TO DELAY THE EMERGENCE OF DRUG RESISTANCE

The traditional approach to the emergence of drug resistance has been the introduction of a new drug; either a completely new drug or one designed to be effective against resistant organisms. This approach has been effective for the past four decades however it has served to concentrate drug development efforts on a small number of well-defined targets and a limited set of compounds[3]. In malaria, for example, the majority of drugs target hemoglobin digestion or nucleic acid synthesis. While there are dozens of drugs they fall into a small number of chemical classes and target a limited set of metabolic pathways. As resistance develops sequentially with the use of each drug, the therapeutic potential of each class diminishes[2].

A parallel strategy has been to combine drugs, either because of synergistic interactions, such as the combination of pyrimethamine and sulfadoxine in Fansidar or atovaquone and proguanil in Malarone[4, 5]. The more recent approach of multidrug therapy, in which artesunate is combined with mefloquine or Fansidar[14, 15]. These approaches are based on the principle that drug resistance to each drug occurs by an independent mechanism and that resistance to multiple drugs will only occur when a single organism or cell contains mutations that lead to resistance to each independent drug. The frequency of this multidrug resistant phenotype will be equal to the product of the frequency of each individual resistance. This strategy is designed to delay the emergence of resistance. In the short term, this strategy has been effective, however, organisms resistant to combination therapy or multidrug therapy do emerge and when they do, often all the drugs used in the therapy are compromised.

STRATEGIES FOR THE REVERSAL OF DRUG RESISTANCE

In Plasmodium falciparum, there is evidence for at least two different mechanisms of drug resistance, the selection of mutation(s) (either single base pair changes or small insertions or deletions) in the target enzyme or the amplification of certain genes associated with drug resistance. In selecting new targets and new drugs, the potential for resistance should be addressed. An alternate approach is to directly target the mechanism of drug resistance and attempt either to prevent the original emergence or to reverse resistance once it has appeared. This type of strategy has been initiated in the cancer chemotherapy field and has focused on the major mechanism of resistance that compromises cancer chemotherapy. The mechanism of this resistance is drug efflux via an ABC (ATP-binding cassette)-transporter[16]. Drug resistance can be modulated by the use of several compounds that appear to inhibit drug efflux. This reversal of drug resistance through the administration of a second compound is the basis for the development of a new type of combination therapy in which one drug in the combination is designed to reverse the resistance or inhibit the efflux of the second drug. Several reversal agents have reached the clinical trial stage in cancer chemotherapy and these trials are underway. In the case of malaria, the potential role

of ABC-transporters in drug resistance was first proposed when chloroquine resistance was reversed in vitro by cotreatment with verapamil and chloroquine[17]. Several ABC-transporters have been identified in P. falciaprum and current work is underway to determine if these are potential targets for this novel strategy for resistance reversal[18]. Additional genes have been implicated in chloroquine resistance, in particular the cg2 locus which encodes a large polymorphic protein[19]

ALTERNATIVES TO NEW DRUGS: VACCINE DEVELOPMENT AND INSECTICIDE IMPREGNATED BEDNETS

Effective antimalarial drugs have been the main tool for the prevention and treatment of acute malaria for the past six decades, however, alternate approaches including the interruption of disease transmission targeting the insect vector or the prevention of initial infection through vaccine development are both being actively pursued in the malaria research community. A brief summary of current thinking in these areas is included below, however, more extensive discussions of these areas can be found at the WHO website (http://www.who.ch) under the Roll Back Malaria link.

VACCINE DEVELOPMENT

Malaria presents special challenges to vaccine development in that, unlike these other infections, malaria induces only partial immunity in those who contract the disease[7]. After multiple bouts of the disease, a person develops enough immunity to prevent severe infection and mortality, but can still become ill. Moreover, even this limited immunity can be maintained only by frequent reinfection. Thus the ideal vaccine—one that could provide full and permanent protection from illness—would have to perform better than natural immunity, an ambitious goal for which there is no precedent. If researchers were to succeed in developing a vaccine that mimics naturally occurring immunity, it could save millions of children's' lives. The mechanism by which people eventually become partially immune to malaria is not understood. It may be related to the complexity of the parasite. Recent work has demonstrated that the malaria parasite expresses a repertoire variant antigen in a single infection. The parasite therefore presents a moving target for the host immune system and multiple exposures to different variant antigens may be required to develop a partially protective immune response[13].

Vaccine strategies have focused on the three major phases of the parasite life cycle, a sporozoite vaccine, a blood stage vaccine and a vaccine directed at the prevention of transmission.[7]. Candidate subunit vaccines are available for all of these targets including peptide-based vaccines, whole recombinant protein vaccines and DNA vaccines. All have shown promising results in animals, and many have been shown to be antigenic in humans, however, only one vaccine has given limited protected efficacy from challenge infection in humans[20]. What we have learned from these initial studies may help in designing the next generation of vaccines

INSECTICIDE IMPREGNATED BEDNETS

Given that an effective vaccine is years if not decades away, and that the best vaccine is likely to have only limited impact in preventing illness, we need to step back and reemphasize both prevention and control. One very promising way to prevent malaria is to use mosquito nets treated with a safe, biodegradable pyrethroid insecticide to protect sleeping children[21]. In the early 1990s, four large-scale, randomized controlled community trials were conducted in four African countries representing different malaria risks— Burkina Faso, Ghana, Kenya and the Gambia— to determine the impact of using treated nets on mortality rates of children younger than five. The three-year trials, conducted under the auspices of the U.N. Development Programme (UNDP), WHO, and the World Bank, involved nearly half a million people and 20 research institutes and donors. The results were dramatic: children's deaths from all causes dropped 15 percent in Burkina Faso, 17 percent in Ghana, 33 percent in Kenya, and 25 percent in the Gambia. The magnitude of the reduction in mortality indicates either that malaria is the most important cause of death in the age groups included in the trials or that preventing malaria in young children somehow helps reduce their likelihood of dying from other diseases. These results suggest that if bed nets were made widely available, they could save the lives of up to 500,000 African children each year.

THE MALARIA GENOME PROJECT: A NEW ERA IN DRUG DISCOVERY

The Malaria Genome Sequencing Project is a cooperative effort and seeks to define every gene in the organism and, armed with this information, to identify new targets for drug and vaccine development[22]. The sequences of chromosome 2 [23] and 3 have been completed and high throughput sequencing is completed for approximately 50% of the genome. The Plasmodium falciparum genome presented a number of technical hurdles, most prominent was the overall high A+T content (80.2% in chromosome 2) requiring adaptation of standard cloning techniques, modification of programs used to assemble the sequence and development of new methods to finish the sequence. The entire genome is approximately 30 Mb in length, arranged in 14 individual chromosomes. Chromosome 2 is just under 1 Mb in length and contains 209 predicted open reading frames and Chromosome 3 is 1Mb in length and contains 215 open reading frames. By comparing gene sequences in the parasite with those of other organisms, homologues were identified for about 40% of the genes with the remaining 60% falling into the class of unidentified open reading frames. This is a significantly higher percentage of unidentified open reading frames than reported in other organisms and this may be due, in part, to the unusually high percentage of non-globular protein domains predicted in P. falciparum open reading frames. An additional contributory factor is certainly that this organism has an unusual and complex life cycle, existing in both insect and human hosts, with multiple forms in each host. Host parasite interactions and immune evasion certainly requires novel genes and proteins.

One of the immediate applications of Malaria Genome Sequencing Project is in the identification of new potential targets for drug development and the results presented from Chromosome 2 and 3 provide several new candidate targets. Of particular interest are enzymes that are drug targets in other organisms that are also present in the malaria parasite or enzymes which are unique to the parasite and not found in the

host; these could serve as targets for new antimalarial drugs. One example which seems promising from Chromosome 2 is the 3-ketoacyl-ACP synthase III (FabH), an enzyme in the Type II fatty acid synthase system, which had previously been restricted to bacteria and plant plastid systems. Additional research will be needed to determine if such putative targets are indeed essential for parasite survival and are amenable to drug development. The sequence gives us direct access to the genes and the research can move directly to the focused effort of target evaluation. Further, comparison of Plasmodium falciparum and related organisms can provide leads to genes of unknown function, which are conserved and thus may play an essential role in parasite survival.

Information from the genome project may also help scientists develop new DNA-based vaccines. For example, new putative surface protein encoding genes have been identified on Chromosome 2 and 3 (e.g., the Rif-1 gene family) and some of these may warrant further investigation as potential vaccine targets. Further, as new protein domains important in cell-cell interaction are identified through biological and immunological analysis, related genes can be readily sought in the sequence database. This will provide immediate information regarding variant and conserved regions useful for vaccine design.

Sequencing and assembly of the remaining twelve chromosomes is underway, with about 40% of the 6500 predicted genes at least partially sequenced. This now permits us to think about future applications, which are now possible with the availability of the entire genome sequence. Of particular interest is the analysis of gene expression at the level of the entire organism through the use of DNA microarray or Serial Analysis of Gene Expression approaches. These approaches have proved extremely powerful in the analysis of Sachyromyces cerevisae gene expression, identifying large groups of genes which respond when yeast are grown under different conditions or in the presence of drugs. It seems reasonable to assume that a similar multigene response of the Plasmodium falciparum parasite will occur as the organism responds to new vectors, drugs or the host's immune response. Previous work has focused on single genes; the availability of the tools provided by the Plasmodium falciparum Genome Sequencing Project would now allow us to take a more integrative approach, analyzing the interaction of several genes in response to a single stimulus. The ability to dissect these kinds of complex responses is likely to bring a whole new dimension to our understanding of the parasite.

Finally, availability of the Chromosome 2 sequence will allow for comparison of populations of parasites both to determine sequence polymorphisms and allelic diversity. Comparison of sequences from recent parasite isolates with the reference strain sequence may provide insight into mechanisms of pathogenicity and virulence. Comparison of gene expression profiles may provide clues to those genes that are essential for growth in the human host but not required for growth in in vitro culture.

Concluding Remarks - Fundamental research into the biology of the parasite and the pathogenesis of disease promises new insight into mechanisms by which the malaria parasite operates and might be defeated. The challenge facing the research community is to bring this knowledge to the treatment and prevention of this disease. Many other diseases such as tuberculosis present similar challenges to the research and world health community and we must find a new paradigm in a skills based collaboration between the public/academic sector and the pharmaceutical/ biotechnology industry to meet this challenge. Every twelve seconds, a child dies of malaria and the hope is that this new initiative will lead to a fundamental and lasting change in that statistic.

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urokinase-type plasminogen activator, 34, 121
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x-ray crystallography, 21, 293; 27, 271

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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
anagrelide hydrochloride	hematological	1997	33, 328
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

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astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
atorvastatin calcium	dyslipidemia	1997	33, 328
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
balsalazide disodium	ulcerative colitis	1997	33, 329
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
brinzolamide	antiglaucoma	1998	34, 318
brodimoprin	antibiotic	1993	29, 333
bromfenac sodium	NSAID	1997	33, 329
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budipine	antiParkinsonian	1997	33, 330
budralazine	antihypertensive	1983	19, 315
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

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butyl flufenamate	topical antiinflammatory	1983	19, 316
cabergoline	antiprolactin	1993	29, 334
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	hypertensive	1988	24, 298
calcipotriol	antipsoriatic	1991	27, 323
camostat mesylate	antineoplastic	1985	21, 325
candesartan cilexetil	antihypertension	1997	33, 330
capecitabine	antineoplastic	1998	34, 319
carboplatin	antibiotic	1986	22, 318
carperitide	congestive heart failure	1995	31, 339
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
cefbuperazone sodium	antibiotic	1985	21, 325
cefcapene pivoxil	antibiotic	1997	33, 330
cefdinir	antibiotic	1991	27, 323
cefditoren pivoxil	oral cephalosporin	1994	30, 297
cefepime	antibiotic	1993	29, 334
cefetamet pivoxil 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
cefoselis	antibiotic	1998	34, 319
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
cerivastatin	dyslipidemia	1997	33, 331
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
choline alfoscerate	nootropic	1990	26, 300
cibenzoline	antiarrhythmic	1985	21, 325

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cicletanine	antihypertensive	1988	24, 299
cidofovir	antiviral	1996	32, 306
cilazapril	antihypertensive	1990	26, 301
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
cinildipine	antihypertensive	1995	31, 339
cinitapride	gastroprokinetic	1990	26, 301
cinolazepam	hypnotic	1993	29, 334
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
cisatracurium besilate	muscle relaxant	1995	31, 340
citalopram	antidepressant	1989	25, 311
cladribine	antineoplastic	1993	29, 335
clarithromycin	antibiotic	1990	26, 302
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
clopidogrel hydrogensulfate	antithrombotic	1998	34, 320
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
delavirdine mesylate	antiviral	1997	33, 331
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
dexfenfluramine	antiobesity	1997	33, 332
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
dolasetron mesylate	antiemetic	1998	34, 321
donepezil hydrochloride	anti-Alzheimer	1997	33, 332
dopexamine	cardiostimulant	1989	25, 312
dornase alfa	cystic fibrosis	1994	30, 298

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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
ebrotidine	antiulcer	1997	33, 333
ecabet sodium	antiulcerative	1993	29, 336
efavirenz	antiviral	1998	34, 321
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
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
entacapone	antiparkinsonian	1998	34, 322
epalrestat	antidiabetic	1992	28, 330
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epinastine	antiallergic	1994	30, 299
epirubicin HCl	antineoplastic	1984	20, 318
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eprosartan	antihypertensive	1997	33, 333
eptazocine HBr	analgesic	1987	23, 334
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

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felbamate	antiepileptic	1993	29, 337
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenoldopam mesylate	antihypertensive	1998	34, 322
fenticonazole nitrate	antifungal	1987	23, 334
fexofenadine	antiallergic	1996	32, 307
filgrastim	immunostimulant	1991	27, 327
finasteride	5α-reductase inhibitor	1992	28, 331
fisalamine	intestinal antiinflammatory	1984	20, 318
fleroxacin	antibacterial	1992	28, 331
flomoxef sodium	antibiotic	1988	24, 302
flosequinan	cardiostimulant	1992	28, 331
fluconazole	antifungal	1988	24, 303
fludarabine phosphate	antineoplastic	1991	27, 327
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flurithromycin ethylsuccinate	antibiotic	1997	33, 333
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
fluticasone propionate	antiinflammatory	1990	26, 303
flutoprazepam	anxiolytic	1986	22, 320
flutrimazole	topical antifungal	1995	31, 343
flutropium bromide	antitussive	1988	24, 303
fluvastatin	hypolipaemic	1994	30, 300
fluvoxamine maleate	antidepressant	1983	19, 319
follitropin alfa	fertility enhancer	1996	32, 307
follitropin beta	fertility enhancer	1996	32, 308
fomepizole	antidote	1998	34, 323
fomivirsen sodium	antiviral	1998	34, 323
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
fropenam	antibiotic	1997	33, 334
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

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glatiramer acetate	Multiple Sclerosis	1997	33, 334
glimepiride	antidiabetic	1995	31, 344
glucagon, rDNA	hypoglycemia	1993	29, 338
GMDP	immunostimulant	1996	32, 308
goserelin	hormone	1987	23, 336
granisetron 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
imiquimod	antiviral	1997	33, 335
incadronic acid	osteoporosis	1997	33, 335
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indinavir sulfate	antiviral	1996	32, 310
indobufen	antithrombotic	1984	20, 319
insulin lispro	antidiabetic	1996	32, 310
interferon alfacon-1	antiviral	1997	33, 336
interferon, β-1a	multiple sclerosis	1996	32, 311
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
irbesartan	antihypertensive	1997	33, 336
irinotecan	antineoplastic	1994	30, 301
irsogladine	antiulcer	1989	25, 315
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam	antiinflammatory	1983	19, 320
isradipine	antihypertensive	1989	25, 315
itopride HCl	gastroprokinetic	1995	31, 344
itraconazole	antifungal	1988	24, 305

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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
lefunomide	antiarthritic	1998	34, 324
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
lepirudin	anticoagulant	1997	33, 336
lercanidipine	antihyperintensive	1997	33, 337
letrazole	anticancer	1996	32, 311
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
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
lornoxicam	NSAID	1997	33, 337
losartan	antihypertensive	1994	30, 302
loteprednol etabonate	antiallergic ophthalmic	1998	34; 324
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatroprotective	1985	21, 329
manidipine hydrochloride	antihypertensive	1990	26, 304
masoprocol	topical antineoplastic	1992	28, 333
mebefradil hydrochoride	antihypertensive	1997	33, 338
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
meloxicam	antiarthritic	1996	32, 312

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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
miglitol	antidiabetic	1998	34, 325
milnacipran	antidepressant	1997	33, 338
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303
misoprostol	antiulcer	1985	21, 329
mivacurium chloride	muscle relaxant	1992	28, 334
mitoxantrone HCl	antineoplastic	1984	20, 321
mizolastine	antihistamine	1998	34, 325
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
modafinil	idiopathic hypersomnia	1994	30, 303
moexipril HCl	antihypertensive	1995	31, 346
mofezolac	analgesic	1994	30, 304
mometasone furoate	topical antiinflammatory	1987	23, 338
montelukast sodium	antiasthma	1998	34, 326
moricizine hydrochloride	antiarrhythmic	1990	26, 305
mosapride citrate	gastroprokinetic	1998	34, 326
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
naratriptan hydrochloride	antimigraine	1997	33, 339
nartograstim	leukopenia	1994	30, 304
nazasetron	antiemetic	1994	30, 305
nebivolol	antihypertensive	1997	33, 339
nedaplatin	antineoplastic	1995	31, 347
nedocromil sodium	antiallergic	1986	22, 324
nefazodone	antidepressant	1994	30, 305
neflinavir mesylate	antiviral	1997	33, 340
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331

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neticonazole HCl	topical antifungal	1993	29, 341
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
olopatadine hydrochloride	antiallergic	1997	33, 340
omeprazole	antiulcer	1988	24, 308
ondansetron hydrochloride	antiemetic	1990	26, 306
orlistat	antiobesity	1998	34, 327
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
paricalcitol	vitamin D	1998	34 327
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
pirarubicin	antineoplastic	1988	24, 309
pirmenol	antiarrhythmic	1994	30, 307
piroxicam cinnamate	antiinflammatory	1988	24, 309
pivagabine	antidepressant	1997	33, 341
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
pramipexole hydrochloride	antiParkinsonian	1997	33, 341
pramiracetam H_2SO_4	cognition enhancer	1993	29, 343
pranlukast	antiasthmatic	1995	31, 347
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
prezatide copper acetate	vulnery	1996	32, 314
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propagermanium	antiviral	1994	30, 308
propentofylline propionate	cerebral vasodilator	1988	24, 310
propiverine hydrochloride	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
pumactant	lung surfactant	1994	30, 308
quazepam	hypnotic	1985	21, 332
quetiapine fumarate	neuroleptic	1997	33, 341
quinagolide	hyperprolactinemia	1994	30, 309
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
rabeprazole sodium	gastric antisecretory	1998	34, 328
raltitrexed	anticancer	1996	32, 315
raloxifene hydrochloride	osteoporosis	1998	34, 328
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
reboxetine	antidepressant	1997	33, 342
remifentanil HCl	analgesic	1996	32, 316
remoxipride hydrochloride	antipsychotic	1990	26, 308
repaglinide	antidiabetic	1998	34, 329
repirinast	antiallergic	1987	23, 341
reteplase	fibrinolytic	1996	32, 316
reviparin sodium	anticoagulant	1993	29, 344
rifabutin	antibacterial	1992	28, 335
rifapentine	antibacterial	1988	24, 310

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
riluzole	neuroprotective	1996	32, 316
rimantadine HCl	antiviral	1987	23, 342
rimexolone	antiinflammatory	1995	31, 348
risedronate sodium	osteoporosis	1998	34, 330
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rivastigmin	anti-Alzheimer	1997	33, 342
rizatriptan benzoate	antimigraine	1998	34, 330
rocuronium bromide	neuromuscular blocker	1994	30, 309
rokitamycin	antibiotic	1986	22, 325
romurtide	immunostimulant	1991	27, 332
ronafibrate	hypolipidemic	1986	22, 326
ropinirole HCl	antiParkinsonian	1996	32, 317
ropivacaine	anesthetic	1996	32, 318
rosaprostol	antiulcer	1985	21, 332
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 mesvlate	antiviral	1995	31, 349
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
seratrodast	antiasthmatic	1995	31, 349
sertaconazole nitrate	topical antifungal	1992	28, 336
sertindole	neuroleptic	1996	32, 318
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline hydrochloride	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
sibutramine	antiobesity	1998	34, 331
sildenafil citrate	male sexual dysfunction	1998	34, 331
simvastatin	hypocholesterolemic	1988	24, 311
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
sparfloxacin	antibiotic	1993	29, 345
spirapril HCl	antihypertensive	1995	31, 349
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	β-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
suplatast tosilate	antiallergic	1995	31, 350
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
tacalcitol	topical antipsoriatic	1993	29, 346
tacrine HCl	Alzheimer's disease	1993	29, 346
tacrolimus	immunosuppressant	1993	29, 347
talipexole	antiParkinsonian	1996	32, 318
tamsulosin HCl	antiprostatic hypertrophy	1993	29, 347
tandospirone	anxiolytic	1996	32, 319
tazobactam sodium	β-lactamase inhibitor	1992	28, 336
tazanolast	antiallergic	1990	26, 309
tazarotene	antipsoriasis	1997	33, 343
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
tirofiban hydrochloride	antithrombotic	1998	34, 332
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
tolcapone	antiParkinsonian	1997	33, 343
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
topiramate	antiepileptic	1995	31, 351
topotecan HCl	anticancer	1996	32, 320
torasemide	diuretic	1993	29, 348
toremifene	antineoplastic	1989	25, 319
tosufloxacin tosylate	antibacterial	1990	26, 310
trandolapril	antihypertensive	1993	29, 348
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
troglitazone	antidiabetic	1997	33, 344
tropisetron	antiemetic	1992	28, 337
trovafloxacin mesylate	antibiotic	1998	34, 332
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
unoprostone isopropyl ester	antiglaucoma	1994	30, 312
valaciclovir HCl	antiviral	1995	31, 352
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
zileuton	antiasthma	1997	33, 344
zidovudine	antiviral	1987	23, 345
zinostatin stimalamer	antineoplastic	1994	30, 313
zolpidem hemitartrate	hypnotic	1988	24, 313
zomitriptan	antimigraine	1997	33, 345
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19, 319
mifepristone		1988	24, 306
lanreotide acetate	ACROMEGALY	1995	31, 345
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
olopatadine hydrochloride		1997	33, 340
repirinast		1987	23, 341
suplatast tosilate		1995	31, 350
tazanolast		1990	26, 309
lodoxamide tromethamine	ANTIALLERGIC OPHTHALMIC	1992	28, 333

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
donepezil hydrochloride	ANTI-ALZHEIMERS	1997	33, 332
rivastigmin		1997	33, 342
gallopamil HCl	ANTIANGINAL	1983	19, 319
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
zileuton		1997	33, 344
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
cefcapene pivoxil		1997	33, 330

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefdinir		1991	27, 323
cefepime		1993	29, 334
cefetamet pivoxil hydrochloride		1992	28, 327
cefixime		1987	23, 329
cefmenoxime HCl		1983	19, 316
cefminox sodium		1987	23, 330
cefodizime sodium		1990	26, 300
cefonicid sodium		1984	20, 316
ceforanide		1984	20, 317
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
flurithromycin ethylsuccinate		1997	33, 333
fropenam		1997	33, 334
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
duteplase	ANTICOAGULANT	1995	31, 342
lepirudin		1997	33, 336
parnaparin sodium		1993	29, 342
reviparin sodium		1993	29, 344
lamotrigine	ANTICONVULSANT	1990	26, 304
oxcarbazepine		1990	26, 307
progabide		1985	21, 331
vigabatrin		1989	25, 319
zonisamide		1989	25, 320
bupropion HCl	ANTIDEPRESSANT	1989	25, 310
citalopram		1989	25, 311
fluoxetine HCl		1986	22, 320
fluvoxamine maleate		1983	19, 319
indalpine		1983	19, 320
medifoxamine fumarate		1986	22, 323
metapramine		1984	20, 320
milnacipran		1997	33, 338
mirtazapine		1994	30, 303
moclobemide		1990	26, 305
nefazodone		1994	30, 305
paroxetine		1991	27, 331
pivagabine		1997	33, 341
reboxetine		1997	33, 342
setiptiline		1989	25, 318
sertraline hydrochloride		1990	26, 309
tianeptine sodium		1983	19, 324
toloxatone		1984	20, 324
venlafaxine		1994	30, 312
acarbose	ANTIDIABETIC	1990	26, 297
epalrestat		1992	28, 330
glimepiride		1995	31, 344
insulin lispro		1996	32, 310
tolrestat		1989	25, 319
troglitazone		1997	33, 344
voglibose		1994	30, 313

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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

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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
bevantolol HCl		1987	23, 328
bisoprolol fumarate		1986	22, 317
bopindolol		1985	21, 324
budralazine		1983	19, 315
bunazosin HCl		1985	21, 324
candesartan cilexetil		1997	33, 330
carvedilol		1991	27, 323
celiprolol HCl		1983	19, 317
cicletanine		1988	24, 299
cilazapril		1990	26, 301
cinildipine		1995	31, 339
delapril		1989	25, 311
dilevalol		1989	25, 311
doxazosin mesylate		1988	24, 300
efonidipine		1994	30, 299
enalapril maleate		1984	20, 317
enalaprilat		1987	23, 332
eprosartan		1997	33, 333
felodipine		1988	24, 302
fosinopril sodium		1991	27, 328
guanadrel sulfate		1983	19, 319
imidapril HCl		1993	29, 339
irbesartan		1997	33, 336
isradipine		1989	25, 315
ketanserin		1985	21, 328
lacidipine		1991	27, 330
lercanidipine		1997	33, 337
lisinopril		1987	23, 337
losartan		1994	30, 302
manidipine hydrochloride		1990	26, 304
mebefradil hydrochloride		1997	33, 338
moexipril HCl		1995	31, 346
moxonidine		1991	27, 330

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nebivolol		1997	33, 339
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
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,	1984	20, 318
osalazine sodium	INTESTINAL	1986	22, 324

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
alpiropride	ANTIMIGRAINE	1988	24, 296
naratriptan hydrochloride		1997	33, 339
sumatriptan succinate		1991	27, 333
zomitriptan		1997	33, 345
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

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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
dexfenfluramine	ANTIOBESITY	1997	33, 332
atovaquone	ANTIPARASITIC	1992	28, 326
ivermectin		1987	23, 336
budipine	ANTIPARKINSONIAN	1997	33, 330
droxidopa		1989	25, 312
pergolide mesylate		1988	24, 308
pramipexole hydrochloride		1997	33, 341
ropinirole HCl		1996	32, 317
talipexole		1996	32, 318
tolcapone		1997	33, 343
lidamidine HCl	ANTIPERISTALTIC	1984	20, 320
gestrinone	ANTIPROGESTOGEN	1986	22, 321
cabergoline	ANTIPROLACTIN	1993	29, 334
tamsulosin HCl	ANTIPROSTATIC HYPERTROPHY	1993	29, 347
acitretin	ANTIPSORIATIC	1989	25, 309
calcipotriol		1991	27, 323
tazarotene		1997	33, 343

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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
benexate HCl	ANTIULCER	1987	23, 328
ebrotidine		1997	33, 333
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

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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
delavirdine mesylate		1997	33, 331
didanosine		1991	27, 326
famciclovir		1994	30, 300
foscarnet sodium		1989	25, 313
ganciclovir		1988	24, 303
imiquimod		1997	33, 335
indinavir sulfate		1996	32, 310
interferon alfacon-1		1997	33, 336
lamivudine		1995	31, 345
neflinavir mesylate		1997	33, 340
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
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

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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
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

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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
atorvastatin calcium	DYSLIPIDEMIA	1997	33, 328
cerivastatin		1997	33, 331
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
anagrelide hydrochloride	HEMATOLOGIC	1997,	33, 328
erythropoietin	HEMATOPOETIC	1988	24, 301
factor VIII	HEMOSTATIC	1992	28, 330
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

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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
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

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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 ß-1a	MULTIPLE SCLEROSIS	1996	32, 311
interferon ß-1b		1993	29, 339
glatiramer acetate		1997	33, 334
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
clospipramine hydrochloride	NEUROLEPTIC	1991	27, 325
nemonapride		1991	27, 331
olanzapine		1996	32, 313
quetiapine fumarate		1997	33, 341
risperidone		1993	29, 344

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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
bromfenac sodium	NSAID	1997	33, 329
lornoxicam		1997	33, 337
alendronate sodium	OSTEOPOROSIS	1993	29, 332
ibandronic acid		1996	32, 309
incadronic acid		1997	33, 335
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
balsalazide disodium	ULCERATIVE COLITIS	1997	33, 329
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