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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. Emphasis is placed on emerging topics in the biological sciences which are expected to provide the basis for entirely new future therapies.

Volume 33 retains the familiar format of previous volumes, this year with 37 chapters. Sections I–IV are disease-oriented and generally report on specific medicinal agents with updates from Volume 32 on antithrombotics, neurokinin antagonists, and antifungal agents. As in past volumes, annual updates have been limited to only the most active areas of research in specifically focused and mechanistically oriented chapters, where the objective is to provide the reader with the most important new results in a particular field. To this end, chapters on topics not reported in at least five years include: serotonin, histamine H3 receptors, sodium channels, epilepsy, phosphodiesterase IV, adenosine, antimicrobial potentiation, B3 adrenergic receptor agonists, and prostanoid receptors.

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 pro-inflammatory kinases, macrophage inhibitory factor, Bcl-2, chemokine receptors as HIV co-receptors, and caspases. 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 predictive toxicology, surface plasmon resonance, high-throughput screening, and antisense oligonucleotides.

Volume 33 concludes with To Market, To Market—a chapter on NCE introductions worldwide in 1997 and chapters on gender-based medicine, chemoinformatics, technology providers and integrators, and two chapters on glossary of terms used in medicinal chemistry and in computational chemistry. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in volumes 1-33 of this series.

Volume 33 completes my 9th and last year as Editor-in-Chief of Annual Reports in Medicinal Chemistry. During this period, it has been my pleasure to work with 13 highly professional section editors and numerous authors whose critical and insightful chapters have contributed to the success of this series.

James A. Bristol
Ann Arbor, Michigan
May 1998

SECTION I. CNS AGENTS

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Chapter 1. Dopaminergic Approaches to Antipsychotic Agents

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Introduction - Schizophrenia is a psychotic disorder of unknown etiology in which patients suffer from a cluster of symptoms which may include both positive (delusions, hallucinations, disordered thoughts, and disorganized speech) and negative (flat affect, anhedonia, social withdrawal, emotional detachment, cognitive deficits, and poverty of speech) symptoms. This disease, which is relatively common (lifetime prevalence rate ~ 1%), usually strikes its victims during adolescence or early adulthood. Since occupational and social function is severely affected, often leading to institutionalization, the cost to society is very high.

The dopamine hypothesis of schizophrenia has provided a framework for understanding the disease and proposing approaches to its treatment. This hypothesis suggests that schizophrenia results from increased dopaminergic neurotransmission and that treatments which decrease dopaminergic function will alleviate psychotic symptoms (1). While the action of most antipsychotic agents can be understood in terms of this hypothesis, alternative approaches to antipsychotics involving glutamate (2), serotonin (3), neuropeptide (principally neurotensin (4) and CCK (5)), muscarinic (6) and adenosine (7) neurotransmitter systems, and the sigma binding site (8) have been proposed. This report will cover dopaminergic approaches to the treatment of schizophrenia, focusing on work published since the topic was last covered in this series (9). A review of antipsychotic patent literature has appeared recently (10).

Dopamine receptors - Cloning studies have identified five dopamine receptor subtypes which can be grouped into two major classes - the D1 receptors, consisting of the D1 and D5 subtypes, and the D2 receptors, consisting of the D2, D3, and D4 subtypes (11,12,13). In a seminal study, the average daily dose of the antipsychotics was found to be well correlated with affinity of the antipsychotics for the D2 class receptors (14). While positive symptoms respond to treatment with D2 antagonists, the negative symptoms are not appreciably affected; moreover, these agents cause side effects which include sedation, extrapyramidal symptoms (EPS), hyperprolactinemia, and tardive dyskinesia which can limit patient compliance (15). New dopaminergic approaches to antipsychotics have targeted specific receptor subtypes or sought to combine dopaminergic activity with activities at other neurotransmitter receptors.

Clozapine and other multireceptorial agents - Clozapine (1) is the first compound to have been identified as an "atypical" antipsychotic, that is, one which is effective in treating both the positive and negative symptoms of schizophrenia without also inducing EPS and hyperprolactinemia seen with the classical, "typical" antipsychotics. While clozapine is a remarkably effective agent, its utility has been tempered by the observation that 1-2% of treated patients develop agranulocytosis, a potentially fatal blood disorder (16). As a result, clozapine is reserved primarily for refractory patients and, in the US, its use is accompanied by mandatory blood monitoring.

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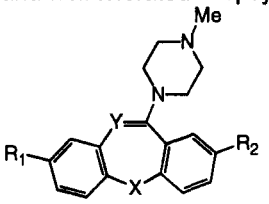
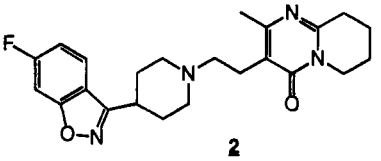
Because of the unique efficacy of clozapine, a great deal of effort has been made to identify compounds with similar clinical efficacy but without its toxicologic problems. Clozapine binds to many different neurotransmitter receptors and it is not yet known which of these interactions are critical for its atypical profile. One approach to identifying new clozapine-like antipsychotic agents is to develop compounds with a similar, broad pharmacologic profile. The Table outlines neuronal receptor binding affinities of clozapine and a number of other antipsychotics either currently in clinical use or under development. Of these multireceptorial agents, olanzapine (3) has a binding profile most similar to that of clozapine (17,18). Like clozapine, upon chronic administration, olanzapine inhibits A10 dopamine firing without affecting A9 firing, suggesting antipsychotic efficacy without EPS liability (19). Indeed, clozapine, olanzapine, and quetiapine (5) are effective antipsychotics, produce minimal EPS and have a surprisingly benign side effect profile in spite of interaction with a number of neuronal receptors (20). Thus, compounds with similar multireceptorial interactions to clozapine are efficacious and well tolerated antipsychotics.



	R_1	R_2	X	Y
1	Cl	H	NH	N
8	Cl	H	CH_2	N
9	Cl	H	O	N
13	OTf	H	NH	N
14	H	Cl	NH	N
15	H	OTf	NH	N



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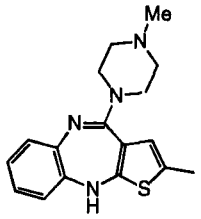

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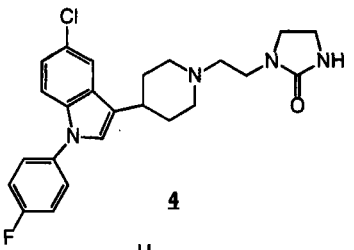

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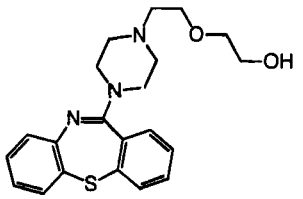

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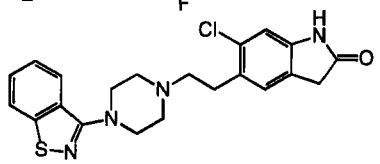

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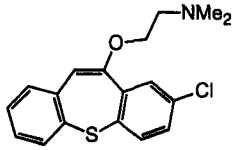

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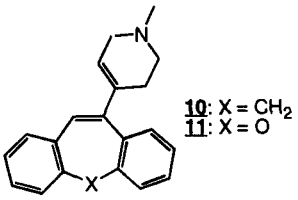
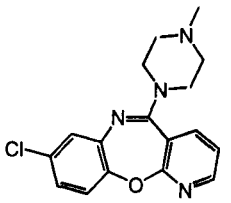
Table: Affinities (K_i, nM) of antipsychotic agents for neuronal receptors (21-23)

Compound	D1	D2	D3	D4.2	5-HT2A	5-HT2C	α1	α2	musc.	H1
Haloperidol	270	1.4	21	11	25	>5000	19	>5000	4670	730
Clozapine, 1	540	150	360	40	3.3	13	23	160	34	2.1
Risperidone, 2	620	3.3	13	16	0.16	63	2.3	7.5	>5000	2.6
Olanzapine, 3	250	17	54	28	1.9	7.1	60	230	26	3.5
Sertindole, 4	210	7.4	8.2	21	0.85	1.3	1.8	1680	>5000	570
Quetiapine, 5	4240	310	650	1600	120	3820	58	87	1020	19
Ziprasidone, 6	330	9.7	7.5	39	0.3	13	12	390	>5000	5.3
Zotepine, 7	84	13	16	39	0.91	2.9	3.4	960	550	3.4

A number of researchers have focused on development of clozapine analogues which retain its pharmacology but are less toxic. A series of clozapine analogues in

which nitrogens in the diazepine and piperazine rings were replaced by carbon or oxygen was prepared and evaluated for affinity at dopamine and serotonin receptors (24,25). Analogues **8** and **9** possessed similar affinities for serotonin receptors as clozapine with **9** having higher dopamine receptor affinity than clozapine and **8** having lower affinity. The distal nitrogen of the piperazine was required for high affinity to dopamine and serotonin receptors. The tetrahydropyridyl analogues **10** and **11** were equipotent with clozapine at the serotonin and dopamine receptors tested. Compound **12** and other pyridine-containing clozapine analogues have similar pharmacology to clozapine (26-28).





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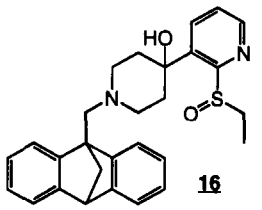

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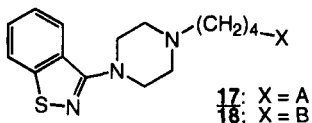
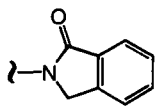
While replacement of the chlorine in clozapine with a trifluoromethanesulfonyloxy group (**13**) led to a significant decrease in receptor affinity, the analogous derivative of isoclozapine (**14**) is GMC1-169 (**15**) which had an *in vitro* and *in vivo* profile predictive of atypical antipsychotic activity (29).

The 9,10-methanodihydroanthracene system of ZD3638 (**16**) represents a departure from the dibenodiazepine nucleus of clozapine. Molecular modeling studies indicated that the aromatic rings of **16** are held in a geometry similar to that seen in both clozapine and conformationally restricted D1 and D2 receptor antagonists (30) and **16** has significant affinity for D1, D2 and 5-HT2A receptors (Ki = 13, 42, 39 nM, respectively).

Combined D2/5-HT2 receptor antagonists - The common anatomical location and functional interactions between the dopaminergic and serotonergic systems, and the high 5-HT2A receptor affinity of a number of atypical antipsychotics including clozapine (22,31,32), led to the proposal that 5-HT2A receptor antagonists may be effective in enhancing the antipsychotic activity and/or ameliorating the side effects of D2 receptor antagonists (3). In particular, it has been proposed that those compounds with a high D2/5-HT2 affinity ratio (D2/5-HT2A > 13) are likely to be atypical antipsychotics (33). A considerable amount of work has been directed toward the identification of compounds with both 5-HT2A and D2 receptor antagonist activity as antipsychotic agents (9,31,34).



17: X = A
18: X = B
19: X = C



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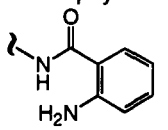

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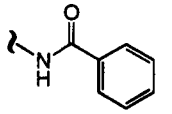
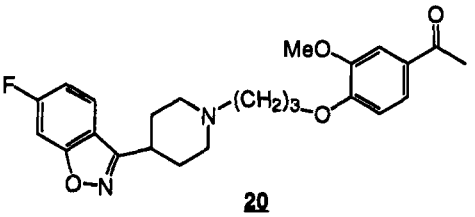


C

Compound **17** has high affinity for the 5-HT2A receptor (Ki = 5.1 nM) and has a D2/5-HT2A ratio of 8.4. It was designed by optimizing the linker connecting a cyclic benzamide moiety, intended to impart D2 antagonist activity, to benzoisothiazol-3-yl piperazine, a 5-HT2 antagonist fragment also present in tiospirone (35,36). It was found that the cyclic amide could be replaced by a benzamide and optimization of the substituents on the benzamide provided 1192U90 (**18**), a high affinity 5-HT2A receptor antagonist (Ki = 3.3 nM) with a D2/5-HT2A ratio of 10 (37-39). An intramolecular hydrogen bond is not critical for the activity of **18** since the unsubstituted amide **19** had similar *in vitro* activity (5-HT2A Ki = 2.1 nM, D2/5-HT2A = 12). The benzamide could also be replaced by heteroaromatic amides and still retain the desired activities (40).

Joining the same benzoisothiazol-3-yl piperazine moiety to an indole fragment intended to mimic the aromatic rings of serotonin and dopamine led to **6** (41).



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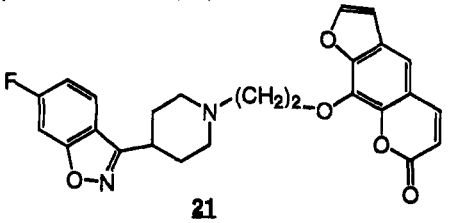
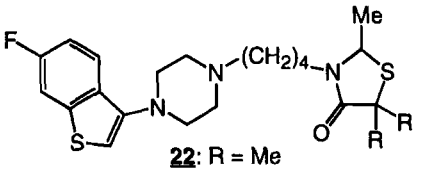
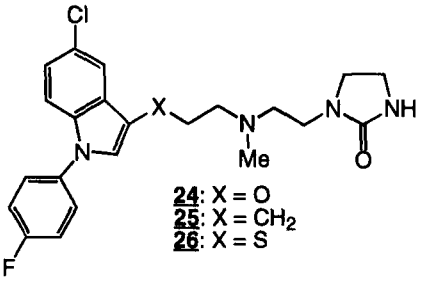


21

The similar 3-(4-piperidinyl)-1,2-benzisoxazole nucleus, present in the 5-HT2 antagonist risperidone (**2**), was incorporated into iloperidone (**20**) which has a D2/5-HT2A ratio of 12 (42,43). The analogous furocoumarin derivative **21** was found to have a D2/5-HT2A ratio of 100 and displayed clozapine-like behavioral activity *in vivo* (44). The SAR of a series of arylpiperazines which contain the 3-butyl-(thiazolidinone) sidechain has been reported. HP-236 (**22**) and **23** display *in vivo* pharmacology indicative of atypical antipsychotic activity (45).



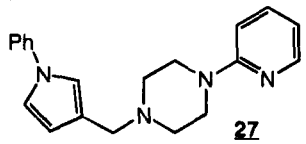
22: R = Me
23: R,R = $(CH_2)_4$



24: X = O
25: X = CH_2
26: X = S

The D2 and 5-HT2A receptor antagonist activity of **4** was retained following replacement of the piperazine ring with the 2-(methylamino)ethoxy group (**24**) but the corresponding carbon and sulfur analogues **25** and **26** lost activity (46). These results were rationalized based on a previously published computational model (47).

D4 selective dopamine receptor antagonists - The high affinity of clozapine for the D4 receptor and the observation that clinically effective plasma levels of clozapine correlate better with its D4 receptor affinity than its D2 receptor affinity (48) have focused attention on the development of selective D4 receptor antagonists as antipsychotic agents. In addition, an increase in the number of D4 receptors in the brains of patients with schizophrenia over normal controls has been reported (49), although this finding has been disputed (50).



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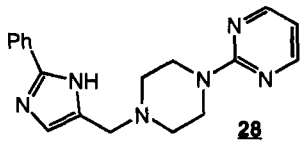

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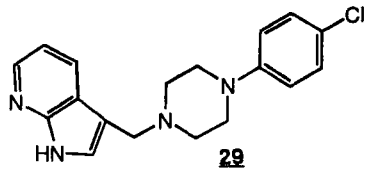

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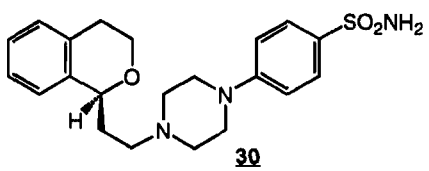
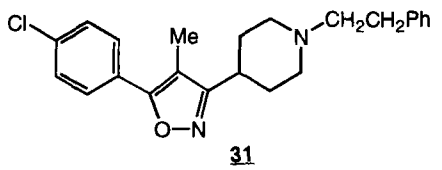


30

A number of structurally related arylpiperazines have demonstrated high affinity and selectivity for the D4 receptor. Compound **27** (Ki = 1.6 nM) was among the first reported to possess significant selectivity for the D4 receptor, but it was not

characterized as either an agonist or antagonist (51). Subsequent refinement of the series led to NGD94-1 (**28**) which showed high affinity (Ki = 3.6 nM), improved selectivity, and antagonist activity *in vitro* at the D4 receptor (52). Compound **28** was active in an antipsychotic model *in vivo* (prepulse inhibition) without causing effects predictive of EPS liability (53). The use of [3H]-NGD94-1 in membrane binding and autoradiographic studies has been reported (54). L-745,870 (**29**) has high affinity (Ki = 0.4nM) for the D4 receptor, is more than 2000-fold selective versus the D2 and D3 receptors and is a full antagonist *in vitro* (55). In spite of achieving high brain levels of drug in pharmacokinetic studies, **29** showed no effect in a number of animal models which have been used to predict antipsychotic activity. U-101387 (**30**) is also an arylpiperazine derivative with D4 receptor selectivity (56). The *S* enantiomer (Ki = 7.2 nM) had higher affinity for the D4 receptor than the *R* enantiomer (Ki = 100 nM) and was a full D4 receptor antagonist *in vitro*. This compound is reported to have excellent pharmacokinetics in monkeys and to be in clinical trials for treatment of schizophrenia (56).

L-741,742 (**31**) and its regioisomeric isoxazole analogue are also reported to be potent and selective D4 receptor antagonists (Ki = 3.5 and 2.5 nM, respectively) *in vitro* (57,58). Elaboration of a lead identified from screening a compound library provided **32** which has high affinity and selectivity for the D4 receptor (Ki = 3.6 nM, D2/D4 = 216). It was characterized as a partial agonist with low efficacy (12%) in an *in vitro* functional assay (59). Ester **33** is a high affinity (Ki = 6 nM) antagonist of D4 receptors *in vitro* with over 1000-fold selectivity versus the D2 receptor (60). The N-acyl nemonapride analogue YM-43611 (**34**) displays good affinity for the D4 receptor (Ki = 5.6 nM) but with only modest selectivity against other dopamine receptors (D2/D4 = 34, D3/D4 = 11). Cleavage of the cyclopropylcarbonyl group would yield a nonselective dopamine antagonist, however the metabolic stability of **34** is not reported (61).



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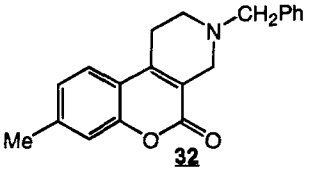

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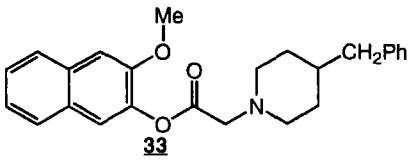

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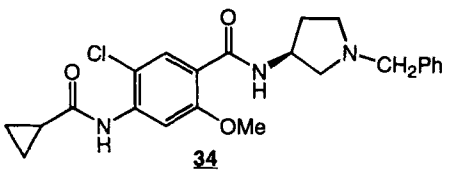

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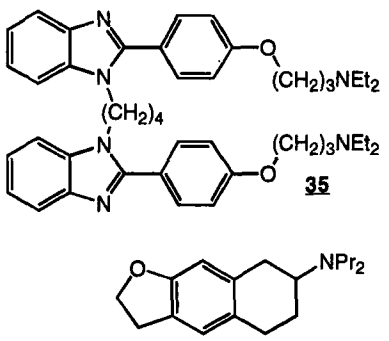
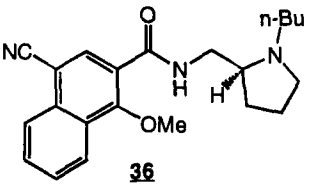
The results of a clinical study of **29** in a four week trial in schizophrenia have been published (62). No efficacy was observed in spite of achieving serum concentrations which were calculated to result in >90% occupancy of the D4 receptor (63). Other D4 receptor antagonists have a different *in vivo* preclinical profile, so clinical trials with those agents are required to demonstrate whether selective D4 receptor antagonists truly lack antipsychotic activity or if this is idiosyncratic to **29**.

D3 selective dopamine receptor antagonists - The D3 dopamine receptor is found primarily in limbic areas of the brain and only at low levels in the striatal regions (64). This is in contrast to the localization of the D2 receptor and implies that D3 selective antagonists may have antipsychotic activity without inducing EPS. The D3 receptor affinities for many compounds, including a number of antipsychotics, have been reported (21,65).



35



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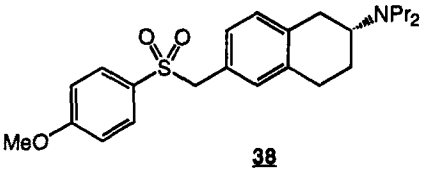
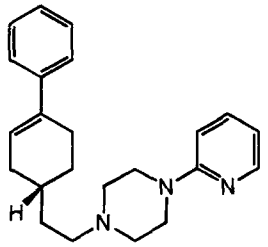


38

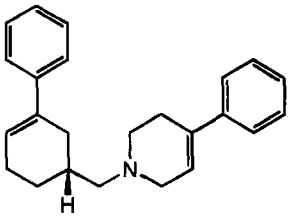
The dimeric benzimidazole PD58491 (**35**, D3: Ki = 19.5 nM, D2/D3 > 100), *l*-nafadotride (**36**, D3: Ki = 0.3 nM, D2/D3 = 10), and the aminotetralin derivative (+)-S14297 (**37**, D3: Ki = 7 nM, D2/D3 = 40) are reported to be D3 receptor antagonists (66-68). GR218231 (**38**) has high affinity and selectivity (D3: Ki = 1 nM, D2/D3 > 400), however it is not reported whether it is an antagonist or an agonist at the D3 receptor (69). Tetracyclic analogues of **37** have shown similar affinity and selectivity to the parent compound (70) but their efficacy was not reported. A series of naphthaleneamides related to **36** has been reported to be agonists and partial agonists at the D3 receptor (71) and a QSAR study of these compounds has appeared (72).

Dopamine D1 receptor antagonists - In three open clinical trials of the D1 antagonist SCH39166 (73,74) in schizophrenia, no appreciable antipsychotic activity was observed (75-77). A clinical study of the D1 receptor antagonist NNC 01-0687 reports efficacy on the negative symptoms of schizophrenia (78).

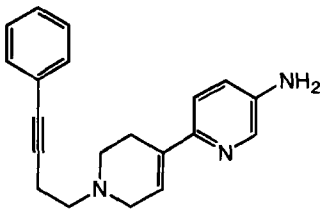
Partial D2 receptor agonists - It has been recognized that dopaminergic transmission may also be decreased by activating dopamine autoreceptors (79). These receptors, located presynaptically on dopamine neurons, are of the D2 class. Activation leads to a decrease in the synthesis and release of dopamine from the nerve terminal. Since D2 receptors are located both presynaptically and postsynaptically, a decrease in dopaminergic transmission will only occur if the presynaptic receptors are selectively activated. A number of compounds, including preclamol (80), terguride (81), and roxindole (82), have been reported with just this sort of selectivity. It is now believed that these compounds are partial agonists at the D2 receptor. Due to the presence of a greater receptor reserve presynaptically than postsynaptically, these partial agonists exert agonistic effects presynaptically while they block postsynaptic receptors (83,84). Preclinical studies have indicated that D2 receptor partial agonists may also alleviate side effects induced by typical neuroleptics. Terguride and preclamol attenuated the catalepsy and hyperprolactinemia induced by haloperidol in rats (85).



42

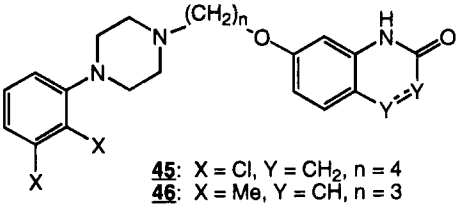


43

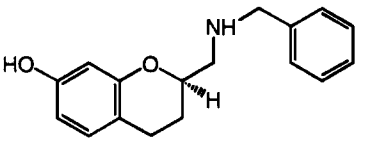


44

From a series of 2-pyridylpiperidines, (-)-PD 135385 (**42**) was identified as a partial agonist at D2 receptors (86). It was effective in decreasing dopamine turnover and decreasing rat locomotor behavior without inducing stereotypic behavior at higher doses. Continued investigation revealed that the 1,4-cyclohexene linker could be replaced with a 1,5-disubstituted cyclohexene provided the ethylene linker was shortened (87). The resulting compound, CI 1007 (**43**), was a partial agonist with 53% the efficacy of the full agonist quinpirole in an *in vitro* second messenger assay (88,89). The conformational rigidity imparted by the cyclohexene of **42** and **43** can be mimicked by an alkyne system, resulting in **44** which displayed 85% agonist efficacy in an *in vitro* functional assay (90).



45: X = Cl, Y = CH_2, n = 4
46: X = Me, Y = CH, n = 3



47

Aripiprazole (**45**) was found to have greater antagonist efficacy than its analogue **46** in a series of *in vivo* assays. It decreased dopamine turnover and blocked apomorphine-induced stereotypy at doses 10-20-fold lower than those required to induce stereotypy (91,92). In an investigation of 2-aminomethylchromane derivatives, **47** was demonstrated to be a high affinity, selective D2 partial agonist (Ki = 0.2 nM) (93).

The efficacy of some D2 partial agonists in treating schizophrenia has been tested clinically, mostly in open label trials with relatively small numbers of patients. Roxindole showed no efficacy in one trial and only marginal efficacy in another, with both trials experiencing a high dropout rate (94). In a double-blind, placebo-controlled study, preclamol was effective, however the antipsychotic activity could not be sustained for longer than a week (95). The investigators propose agonist-induced receptor desensitization for this tolerance. Whether the transient activity of these agents can be avoided through a modified dosing regime, or is inherent to the mechanism, remains to be seen.

Conclusions - While many recent approaches to treating schizophrenia are based on the dopamine hypothesis, improved understanding of dopamine receptor molecular biology, pharmacology, and physiology has led to new hypotheses about antipsychotics. The discovery and clinical study of compounds with better defined pharmacology will allow the testing of these hypotheses. It is hoped that this research will lead to new antipsychotics which not only treat the positive and negative symptoms of schizophrenia while minimizing side effects, but also address the cognitive and mood disorders associated with the disease.

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Chapter 2. Novel Molecular Approaches to Analgesia

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Introduction - Identification of novel compounds which more effectively treat both acute and chronic pain states, and which lack side effects associated with current therapies remains a major challenge in biomedical research (1). At present, pain management continues to rely on nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen, opioids and adjuvant analgesics that include local anesthetics, anticonvulsants and tricyclic antidepressants. Analgesia research over the last several decades has focused largely on identifying safer NSAIDs (resulting in a plethora of classical cyclo-oxygenase (COX) inhibitors, and more recently COX-2 inhibitors) and safer opioids. Efforts directed toward identifying safer opioids are prominently reflected in the last two reports on analgesics in *Annual Reports in Medicinal Chemistry* (2, 3). To date, efforts in analgesia research have met with mixed results in terms of improving on existing therapies.

In the last five to ten years, advances in neurobiology, and development of more sophisticated animal models for clinical pain have led to a paradigm shift in understanding of pain mechanisms. It is now appreciated that all pain states are not the same (4), and that tissue and nerve injury induces phenotypic changes (neuronal plasticity) in pain pathways in the nervous system (4 - 8). These become sensitized peripherally and/or centrally at the level of the spinal cord, resulting in altered processing of noxious and non-noxious sensory information. Alterations in at least 15 distinct neurotransmitters or neuromodulators in the spinal cord, in the descending and ascending pathways associated with pain perception, and/or in the peripheral nervous system have been implicated in central and peripheral sensitization, revealing a range of novel molecular targets for analgesic drug development.

This report highlights centrally acting analgesics like the opioids, as well as the latest advances in the NSAIDs, with information largely limited to the clinical validation of novel, potential near-term analgesics. Additional attention is given to other newly emerging molecular targets that may represent major advances both in our understanding of pain processing and in identifying potential novel treatment options.

Opioids - A major effort has been undertaken over the past 20 years to develop opioid receptor ligands that, because of their receptor subtype selectivity, would be free of the dependence, tolerance, immunosuppression, respiratory depression and constipation associated with traditional opioids like morphine (9, 10). This has been predicated on the cloning and expression of three major opioid receptor subtypes (μ-, δ- and κ-) which are coupled to inhibitory G-protein (pertussis-toxin sensitive) transduction mechanisms. Moreover, each of these opioid subtypes have been further subdivided into putative subtypes, and an "orphan" member of this family, ORL, has also been described (11). To date, the search for improved opioids has been an elusive one, with considerable basic science and clinical trial expenditure, and with success limited to incremental improvements. Nonetheless, efforts are still continuing to identify such compounds, and opioids prevail as the major class of centrally-acting analgesics (3). Tramadol (1) is the most recent opioid introduced to the US market (March, 1995). It is a weak (approx. 2 μM) opioid receptor agonist and monoamine uptake blocker that was in the European market for 15 years prior to US introduction. The rapid uptake of this compound into the market can be attributed to the fact that, unlike most opioids, it is not scheduled. However, the compound is under extensive

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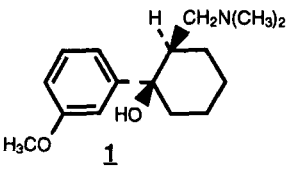
post-marketing surveillance for abuse potential (12), with FDA discussions evaluating the need for it to be scheduled in the near future (13).

The κ-agonists spiradoline and enadoline have been abandoned due to dose-limiting dysphoria in trials of post-surgical pain (14). One trend that seems to be emerging is to target peripheral sites of action in order to limit dependence liabilities, respiratory depression and other CNS side-effects. Opioid agents still at an active stage include (14): ADL 2-1294 a topically active formulation of the μ-receptor agonist, loperamide, targeted for use in burns and abrasions; BCH 3963, a μ-receptor agonist; asimadioline (EMD 61753, **2**), a peripherally active κ-receptor agonist targeted for use in inflammatory hyperalgesia; and other κ-receptor agonists, including apadoline (RP 60180, **3**); TRK-820 and SB 205588 (**4**).



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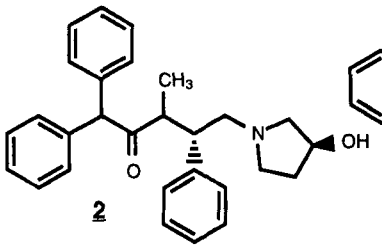

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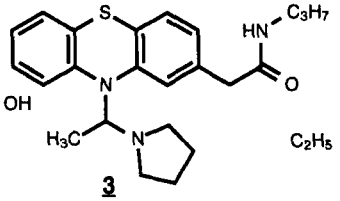

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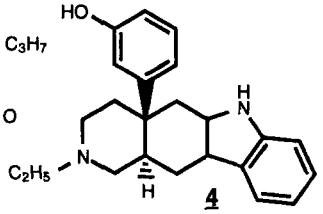

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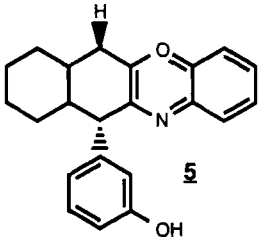
More recent preclinical results indicate that δ-opioid agonists may be safe and effective analgesics (15). Compound **5** (TAN-67; δ K_i = 1.1 nM, μ K_i = 2320 nM, κ K_i = 1800 nM) is representative of these efforts (15). In addition, there has been considerable focus on developing alternative (e.g. intranasal) and delayed release dosage forms of traditional opioids. More than 50% of the near-term pain management pipeline is represented by this category (16).



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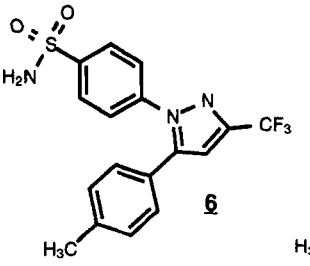
COX-2 Inhibitors - NSAIDs act by blocking the synthesis of prostaglandins *via* the COX pathway (17), diminishing the peripheral sensitization leading to hyperalgesia. Currently available NSAIDs are nonselective inhibitors of both known COX isoforms: COX-1 which is a constitutive enzyme, and the more recently discovered COX-2, which is induced in inflammatory conditions (18, 19). Evidence is emerging that the analgesic and anti-inflammatory effects of the current NSAIDs are mediated by COX-2 inhibition, while their adverse effects (e.g. gastrointestinal lesions and bleeding) may be mediated by COX-1 inhibition (20), providing the rationale for developing selective COX-2 inhibitors as safer analgesic and antiinflammatory agents. An intensive search has lead to the identification of a variety of novel chemical series of selective COX-2 inhibitors, which have been recently reviewed in *Annual Reports in Medicinal Chemistry* (21). Several selective COX-2 inhibitors are currently in clinical development (22), the most advanced being celecoxib (**6**, SC 58635) and MK-966 (**7**). Celecoxib is 375-fold selective for COX-2 (IC_{50} = 40 nM) *versus* COX-1 *in vitro* (23), while MK-966 shows >1000-fold selectivity for COX-2 in cell-based assays (21). Both agents have shown analgesic effects in patients after dental extraction and in short-term osteoarthritis trials (24 - 28). In addition, celecoxib has shown efficacy in short-term



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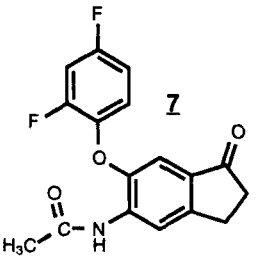

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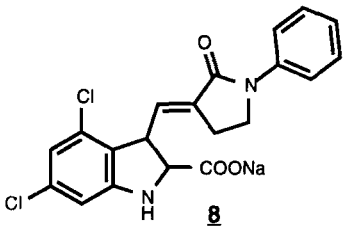
trials in rheumatoid arthritis (24, 25, 29). Data from the dental pain trial indicated a ceiling effect for the analgesic effects of MK-966, as is characteristic for current NSAIDs (30). MK-966 showed markedly improved gastric tolerance compared to aspirin and ibuprofen (24), while celecoxib also showed an improved side effect profile with respect to both gastric tolerance and platelet aggregation (24, 29).

Excitatory Amino Acid Receptor Modulators - The excitatory amino acid, glutamate, plays a key role in processes related to chronic pain and pain-associated neurotoxicity, acting through N-methyl-D-aspartate (NMDA) receptors. In chronic and intractable pain, the NMDA antagonist, (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid ((±)-CPP), abolished the process of central sensitization (31). Other NMDA receptor antagonists like MK-801 (discontinued), ketamine, memantine and dextromethorphan have antinociceptive activity in a variety of animal model systems (32). The major issue with this class of compounds is their psychotomimetic effects that include both dysphoria and cognitive impairment. GV 196771A (**8**) is a compound which modulates NMDA receptor function by blocking the glycine binding site of the NMDA receptor complex (33). Compound **8** is active in animal models of nociception (33). At doses 3- to 10-fold below those eliciting CNS side effects, NMDA antagonists can provide opioid sparing effects and may prevent the tolerance related to prolonged opioid use (32). Late stage clinical trials of a dextromethorphan/morphine combination are ongoing. Antagonists of the kainic acid subtype of the glutamate receptor include LY 293558 (**9**) which is active when delivered at a dose of 1.2 mg/kg in a capsaicin model of allodynia/ hyperalgesia (34).



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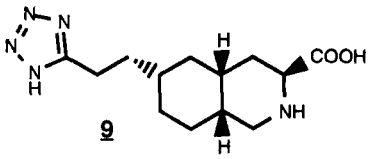

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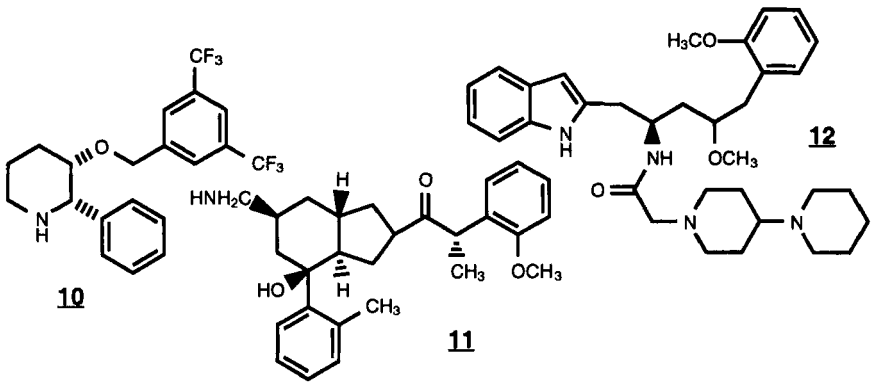
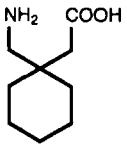
Serotonergics - Several of the many serotonin (5-HT) receptor subtypes (35) have been implicated in modulating pain processing. So far, two areas of pain research have seen particular progress with respect to identifying viable pain management therapies *via* modulation of serotonin receptors: 5-HT1-like receptor subtypes for migraine headache, and 5-HT3 receptors for visceral pain disorders. For migraine, it has been reported that both 5-HT1F and 5-HT1B/D agonists would be useful to treat the neurogenic inflammation associated with trigeminal activation (36), while 5-HT3 receptor antagonists may have utility in modulating visceral sensory information (37). A report of the latest chemistry developments in serotonin receptor modulation appear in this volume (Chapter 3).

Neurokinin Receptor Antagonists - It is well established that the tachykinin, Substance P, plays a major role as a nociceptive transmitter in pain processing as well as in the plasma extravasation involved in migraine. Substance P interacts with the tachykinin receptor family that includes NK-1, NK-2 and NK-3 receptors, an area that has been recently reviewed (38). A number of tachykinin (neurokinin) antagonists have been evaluated in clinical trials for a variety of disease states including pain, with somewhat disappointing results. This may be due to internalization of the receptor after activation, to a nociceptive cascade where Substance P activates a tyrosine kinase to phosphorylate the NMDA receptor evoking neuronal hypersensitivity, or to the inability to interrupt the actions of other nociceptive transmitters conveying "painful" stimuli to the brain. Several, but not all, of the neurokinin antagonists appear to have limited bioavailability. L-733060 (**10**) is a potent NK-1 antagonist that seems to

overcome the bioavailability issues, enter the CNS and inhibit pain-related behavior (39). Other representative NK-1 antagonists (38) include an erispant derivative (K_i = 0.3 nM, 11); and lanepitant (K_i = 0.15 nM, 12). These compounds are, in general, very effective in models of NK-induced pain, but have not shown consistent promise in clinical trials in pain models that are not predominantly mediated by substance P release.



Calcium Channel Modulators - Compounds that alter cell membrane hyperexcitability by altering Ca^{2+} ion channel function have analgesic activity. Ziconitide (SNX-111) is a 25 amino acid peptide ω-conotoxin, isolated from snail venom, that acts as an N-type calcium channel antagonist. It is currently in trials for severe cancer pain, but can be given only *via* the intrathecal route (40). Gabapentin (13), which is approved for use as an anticonvulsant, is used extensively off-label to treat neuropathic pain due to anecdotal evidence of its effectiveness. Gabapentin was thought to be a selective GABA modulator, but evidence for a direct effect on GABAergic function has not been forthcoming. Instead, gabapentin, and a second generation analog, CI-1008 ((*S*)-isobutylGABA)), produce their actions by interacting with the α2δ subunit of the voltage sensitive calcium channel [41].



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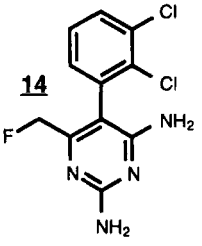
Sodium Channel Blockers - Overexpression or abnormal activation of novel membrane sodium channels has been reported on sensory neurons following inflammation and neuropathy (42 - 44). Tetrodotoxin-sensitive sodium channels are overexpressed in large axons following nerve injury (43). In addition, tetrodotoxin-resistant sodium channels, which are found in many small nociceptive neurons (45), appear to be upregulated by nerve growth factor (NGF), prostanoids and other inflammatory mediators (46 - 48). Upregulation of both PN1-type sodium channels and brain type III sodium channels has also been reported following nerve injury and inflammation (43, 49). Sodium channel blockade may account for the effects of a number of agents currently used in the management of neuropathic pain, including certain local anesthetics (lidocaine, tocainide), anticonvulsant drugs (phenytoin, carbamazepine, lamotrigine), and antiarrhythmic agents (mexilitine). Novel sodium channels are currently being targeted for the development of analgesic agents which might have an improved side effect profile over the exisiting agents due to greater selectivity. The most advanced novel sodium channel blocker is BW4030W92 (14) which is reported to effectively reduce the hypersensitivity accompanying peripheral tissue and nerve injury without changing baseline (non-injured) nociception in preclinical models (50). Recent developments in sodium channels are reviewed further in Chapter 6 of this volume.



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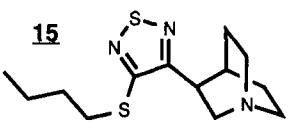
Muscarinic Agonists - Agonists at the various G-protein coupled muscarinic receptors (M_1-M_4) have potent analgesic activity, although this is frequently confounded by typical muscarinic side effects including pronounced effects on GI motility. Vedaclidine (NNC 11-1053, LY297803, **15**) is the most advanced of these agents, having M_1 agonist and M_2/M_3 antagonist activity (51). ET-142 and SS-20 (**16**) are two other muscarinic agonists at the preclinical stage (52).



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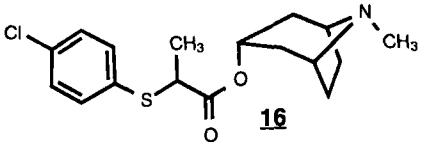

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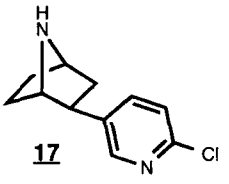
Bradykinin Antagonists - Bradykinin is an important inflammatory mediator, which can activate C- and Aδ- nociceptive afferents, and which appears to play a role in inflammatory pain and hyperalgesia (53). Two subtypes of bradykinin receptors have been identified, B_1 and B_2 (54, 55). The B_2 receptor on neurons appears to play a major role in the early stages of inflammation, while the B_1 receptor, which is upregulated by cytokines, plays a more prominent role in maintaining hyperalgesia (56). A series of small linear and cyclic peptides derived from the carboxy-terminal fragment of the potent B_2 receptor antagonist, HOE 140 (57) have been described, and suggested as leads for the design of nonpepetide ligands of the B_2 receptor (58). A further report describes a series of peptidic mixed B_1/B_2 antagonists with long duration of *in vivo* activity, which have led to the design of potent nonpeptide BK antagonists (59).

Cholinergic Channel Modulators (ChCMs) - Nicotine produces many of its actions in the body by activating different nicotinic acetylcholine receptors (nAChRs) which are ligand - gated ion channels. ChCMs are a broad class of compounds that interact with nAChRs and include nicotinic agonists, nicotinic antagonists, and allosteric modulators (60). Reports indicating that nicotine may have analgesic activity date back to the early 1930s. However, it was not until the discovery of the frog alkaloid, epibatidine (**17**) by Daly (61), that the tools were available to more precisely characterize this novel approach to pain modulation. Epibatidine is 100-200 times more potent than morphine as an analgesic (61). It acts *via* nAChRs, is not antagonized by the opioid receptor antagonist, naloxone, and has very high affinity (K_i = 0.05 nM) for the major nAChR subtype in the brain, $\alpha4\beta2$ (62). This alkaloid is, however, non-selective with regard to its actions at a number of nAChRs including the ganglionic ($\alpha3\beta x$) and neuromuscular ($\alpha1\beta1\delta\gamma\varepsilon$) subtypes, as a consequence of which there is a very narrow window between the beneficial analgesic actions of the compound and its toxic and ultimately lethal effects on the cardiorespiratory system. ABT-594 (**18**), which has a K_i = 0.05 nM at $\alpha4\beta2$, is an (*R*)-azetidine bioisostere of nicotine that retains the analgesic actions associated with this class of compound while showing a reduced propensity towards the toxic side effects seen with epibatidine (62). ABT-594 is approximately 70 times more potent than morphine in a spectrum of acute and chronic nociceptive models and, unlike morphine, shows no evidence of tolerance or opioid-like dependence liability, nor does it have effects on respiration or GI motility (62). This compound is currently in clinical development for the treatment of acute and chronic pain disorders. DBO-83 (**19**) with a K_i = 4 nM at



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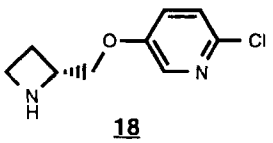

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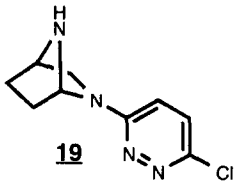

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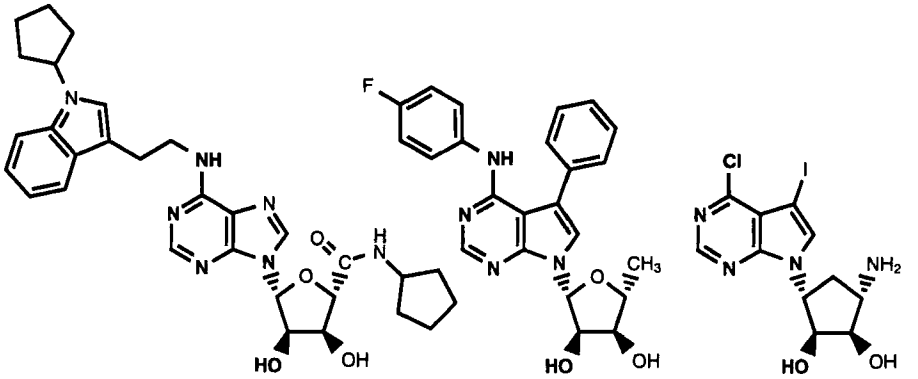
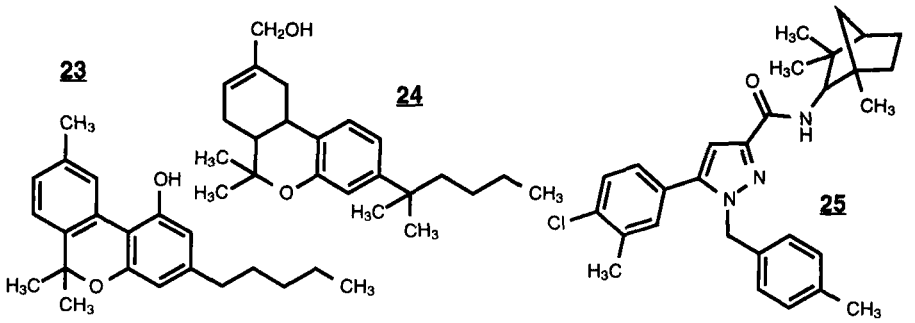
α4β2 (63), and AG-4 (64) represent other novel ChCMs that have shown antinociceptive activity in preclinical pain models.

Adenosine Analogs - The endogenous purine nucleoside, adenosine (ADO), has neuromodulatory, antinociceptive and antiinflammatory properties which are mediated by activation of specific cell-surface receptors (A_1, A_{2A}, A_{2B}, A_3 subtypes) on a variety of cell types (65, 66). ADO analogs produce antinociception in a broad spectrum of animal pain models (67). The pharmacology of these responses suggests that the spinal cord is a key site for antinociception by ADO, and that the A_1 receptor subtype is the primary mediator of the antinociceptive effects of ADO (68). Several approaches to modulating ADO for analgesic benefit are under investigation. ADO itself, administered as an intravenous infusion at doses below those having effects on the cardiovascular system, has shown clinical benefit in human pain states, including post-operative and neuropathic pain (69, 70). In addition, the direct-acting ADO A_1 receptor agonist GR79236 is in clinical trials as a potential treatment for pain (71). A series of mixed A_1/A_{2A} receptor agonists, exemplified by UP-202-32 (**20**), have also been reported to have antinociceptive activity in animal models (72). Inhibitors of the ADO-metabolizing enzyme ADO kinase, which prevent ADO reuptake, therefore amplifying the concentrations and actions of ADO in the extracellular space, represent an alternative approach. Several series of nucleoside-based AK inhibitors with IC_{50} values in the nanomolar range (73 - 75) have been reported, including GP3269 (**21**), and A-127157 (**22**).

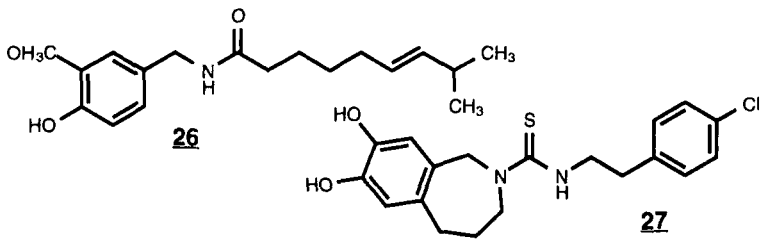


20 **21** **22**

Cannabinoids - While tetrahydrocannabinol (THC) in cannabis is responsible for the euphoria associated with marijuana use, another component, cannabinoldiol (**23**) is a powerful peripheral analgesic (76). Based on the two known types of cannabinoid receptors, CB1 and CB2, the latter not found in neuronal tissues, CB2 ligands have become pharmaceutical targets to avoid producing compounds with CNS effects similar to cannabis. The CB1 receptor inhibits adenyl cyclase activity through activation of Gi protein. The low overall sequence homology (44 %) between CB1 and CB2 suggests it will be possible to identify subtype selective ligands, and has led to the growing interest in the therapeutic application of the cannabinoids (76). Presumably, antinociception can be produced in rodents at peripheral CB2 receptors, and at spinal and supraspinal sites *via* CB1 receptors (77). A very potent cannabinoid lacking a phenolic hydroxy substituent (**24**) has been shown to have high affinity for the CB2 receptor (78). More recently, SR 144528 (**25**), which has CB2 K_i= 0.6 nM and CB1 K_i = 400 nM, was reported as the first highly potent, selective and orally active CB2 receptor antagonist (79). The medicinal chemistry of cannabinoids has been reviewed recently (80).

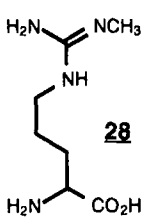


Vanilloid Receptor Modulators - Capsaicin (**26**), is the ingredient in hot chili peppers that elicits an intense burning sensation by activating sensory neurons sensitive to thermal stimuli. Creams containing capsaicin have been used to treat neuropathic pain states, in effect to desensitize nociceptive pathways by hyperexcitation. A rat vanilloid-1 (VR1) receptor has recently been cloned (81). The receptor is sensitive to the agonist, capsaicin (**26**) and to resiniferatoxin, as well as the antagonist, capsazepine (**27**). The VR1 receptor is a non-selective cation channel related to the *Drosophila* retinal protein, TRP, which is thought to be involved in regulating calcium entry into cells that have depleted calcium stores. VR1 is a protein of 838 amino acids containing six transmembrane domains with a short hydrophobic stretch between transmembrane regions 5 and 6. Cells transfected with VR1 show an increase in calcium levels in response to elevated (45 °C) temperatures. Changes in pH, which have previously been implicated in capsaicin receptor activation, were able to potentiate capsaicin-evoked changes in calcium current flow. Northern blot analysis shows that VR1 is expressed in trigeminal and dorsal root ganglion, but not in the nodose ganglion which contains cell bodies of visceral nociceptors, and not in the hypothalamus, which is involved in thermoregulation. This suggests that either VR1 is expressed at levels below detection, or it raises the intriguing potential that distinct VR subtypes may subserve functions related to core temperature regulation, somatic sensations and viscero-sensory control. While efforts to develop more stable analogs of capsaicin, e.g. NE-21610 (nuvanil), have been discontinued due to severe hypothermic effects, the possibility that subtypes of vanilloid receptors regulate these activities may renew interest in the vanilloid receptor area (82).



Nitric Oxide Synthase Inhibitors - Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to produce nitric oxide (NO), which functions as a second messenger in a variety of physiological processes (83, 84), including neurotransmission and inflammation. Three isoforms of NOS have been identified: the constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS), and a cytokine-inducible NOS (iNOS). NOS is upregulated in DRG neurons following nerve injury (85). A role for NO has been described in the thermal hyperalgesia of neuropathic pain (86) and in prolonged chemical nociception (87). In addition, recent studies with the classical NOS inhibitor, L-N^Gmethylarginine (**28**) in patients suggest a role for NO in migraine pain (88).

Early attempts to prepare selective NOS inhibitors produced a series of L-arginine and L-citrulline analogs, as well as non-amino acid isothioureas, amidines, guanidines and a number of nitrogen-containing heterocycles, which have been reviewed (84, 89). More recently, N^{ω}-propyl-L-arginine (**29**), and a series of substituted N-phenylisothioureas, such as (**30**) have been described as selective nNOS inhibitors (90, 91). In addition, a series of N^{ω}-nitroarginine and phenylalanine-containing dipeptides and dipeptide esters have been described, with up to three orders of magnitude selectivity for nNOS over iNOS, although selectivity for nNOS over eNOS was only 2- to 5-fold (92).



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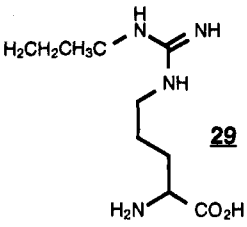

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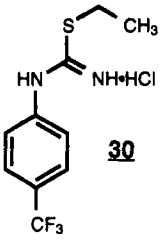

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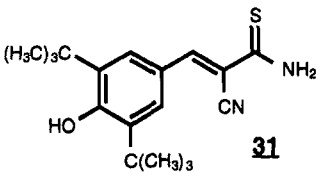
Growth Factors - The role of growth factors in nociceptive processing is an emerging field of research. Administration of NGF (nerve growth factor) to rodents leads to hyperalgesia, while animals with an NGF knockout are insensitive to pain. NGF-mediated signal transduction appears to be involved in inflammatory pain states (93). A NGF antagonist, AG-879 (**31**) is currently in development (94).



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Conclusions - The explosion in knowledge related to the molecular events and pathways involved in pain processing have provided a major impetus to understanding pain and to devising new therapeutic approaches to its treatment. With increasingly well-established and predictive animal models of nociception available to examine the role of these targets in pain, the availability of human clinical pain models and the increasing availability of compounds with novel mechanisms of action, it is anticipated that research efforts will continue to develop the understanding of the discrete events associated with pain, yielding novel analgesic agents early in the 21st century.

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Chapter 3. Latest Developments in Serotonin Receptor Modulation

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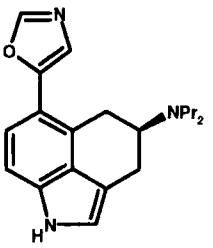
Introduction - The important neurotransmitter serotonin (5-HT) was identified around 50 years ago after it was isolated from whole blood and shown to have vasoconstrictor properties (1). Its identification in brain led to immediate speculation that it could be involved as a mediator in psychiatric disorders (2) and there is now substantial experimental evidence to demonstrate that this hypothesis was correct. The role of 5-HT in appetite, sleep, mood control, thermoregulation and pain has been extensively reviewed (3-5). The multiple actions of 5-HT are mediated through 14 different receptors and these have been subdivided into 7 distinct groups on the basis of operational pharmacology, sequence analysis and transduction mechanisms; it is now clear that all 14 human receptors are encoded by different genes (6-8). Many compounds with mixed 5-HT receptor binding profiles have been reported but this review is intended to summarise recent advances in more receptor specific modulation. For this reason, non-selective agents and combination therapies with serotonin re-uptake inhibitors are not included.

5-HT1A Receptors - Progress in 5-HT1A receptor modulators was reviewed in 1995 (9). The CNS localisation of 5-HT1A receptors implicates their involvement in a number of behavioural processes. They are located presynaptically on cell bodies in the raphé nuclei where they mediate autoinhibitory control of 5-HT neurotransmission, as well as post-synaptically in limbic areas and frontal cortex where they mediate neuronal inhibition (10-14). Buspirone is a full agonist at pre-synaptic and a partial agonist at post-synaptic 5-HT1A receptors (15). Its introduction as an anxiolytic in the mid 1980s prompted the development of a number of other "spirones" such as gepirone, ipsapirone and tandospirone (16-19). 5-HT1A receptor agonists are still being developed as potential anxiolytic/antidepressant agents. These include alnespirone, sunepitron, MKC-242 and ebalzotan (20). BAY x 3702 is being developed as a neuroprotectant (21). A review of buspirone's current clinical pharmacology and therapeutic applications was recently published (15). Preclinical studies have continued with reports of more selective agents such as **1** (LY301317) which displaces [^{3}H]-8-OH-DPAT binding to 5-HT1A receptors in rat hippocampus with Ki of 0.26nM and shows > 200 fold selectivity over 9 other receptors tested. *In vivo*, **1** demonstrated 5-HT1A receptor agonist activity and was able to induce lower lip retraction and flat body posture in rats at 3mg/kg p.o. (22). In pidgeons and rats, **1** increased the rate of punished responding without affecting unpunished responding, indicative of a non-sedative anxiolytic profile. In the forced swim test in rats, **1** significantly decreased immobility time at 1 and 3 mg/kg s.c. in common with a number of antidepressants agents. New benzocycloalkyl piperazines **2**, **3**, and **4**, have recently been reported as selective 5-HT1A receptor agonists with profiles of activity similar to buspirone. In rat hippocampal membranes the (-) enantiomers of **2** and **3** had Ki



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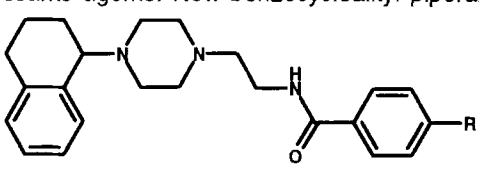
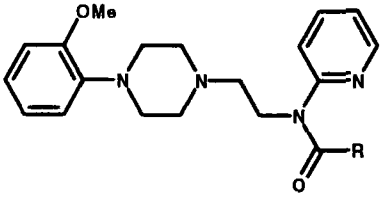


R
2 H
3 F
4 Cl

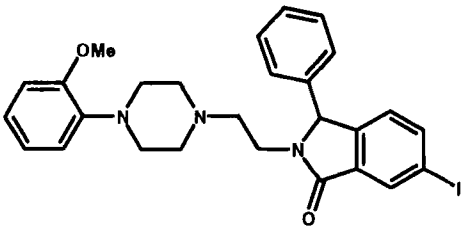
0065-7743/98 $25.00

values for the 5-HT1A receptor of 0.4 nM and 0.5 nM respectively. All three analogues were active *in vivo* in the rat lower lip retraction model of central 5-HT1A receptor function with minimum effective doses of around 2.5 mg/kg i.p. (23).



	R
5	cyclohexyl
6	4-PhF
7	4-PhI

Few selective, silent 5-HT1A receptor antagonists have been identified and there are no reports of their utility in the clinic. WAY-100635, **5** was the first truly selective agent, showing no intrinsic efficacy at either pre- or post-synaptic 5-HT1A receptors (24). Its affinity for human 5-HT1A receptors is sub-nM and it has proved a useful chemical tool for evaluation of 5-HT1A receptor function. It was recently radiolabelled [carbonyl-11-C] and used as a PET ligand for mapping 5-HT1A receptors in human brain (25). More recently, two structurally related analogues, **6** and **7** were shown to have similar profiles (26,27). An attempt was made to develop [125-I]-p-MMPI as a radioligand for *in vivo* imaging of 5-HT1A receptors with SPECT but this was unsuccessful because of rapid *in vivo* metabolism. Related iso-indolones, **8**, and **9** were potent 5-HT1A ligands (Kis 0.07 and 2.5 nM respectively). Their radioiodinated counterparts showed improved metabolic stability over [125-I]-p-MMPI, but they displayed low specific binding to 5-HT1A receptors *in vivo* (28).



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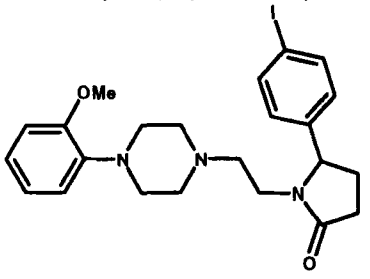
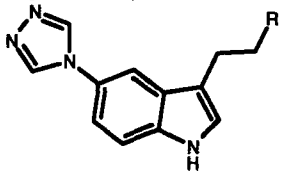


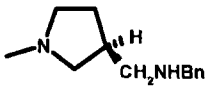
9

5-HT1B/1D Receptors- The 5-HT1B/D receptors were originally subdivided on the basis of their pharmacology but have recently been reclassified (7). Functional distinction between these receptors is still under extensive investigation and is now just beginning to be elucidated with the identification of selective agents, so they are considered together. Several 5-HT1B/1D receptor agonists have been identified as antimigraine drugs. These were reviewed in last year's volume (29) and therefore only reports during the last year are included here.



10, R = NMe_2

11, R =



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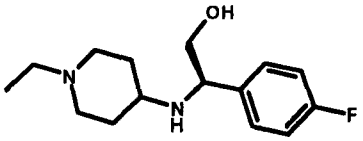
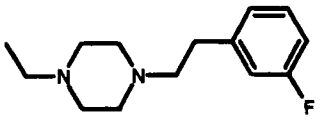
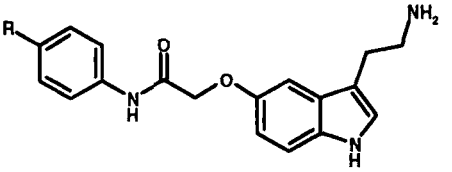


13, R =



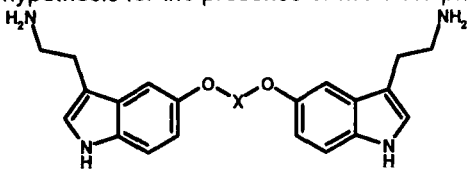
Two further papers have appeared based on the 5-heterocyclic analogue **10** (L-741,604). In the first paper (30), the N-dimethyl group of **10** was replaced by pyrrolidine **11** (L-760,790) and methylenepiperidine **12** (L-772,405). In both series, amino substitution gave h-5-HT1D full agonists of sub-nM affinity which were ≥100

fold selective over h-5-HT1B. In the second paper (31), the piperidine is replaced by substituted phenethylpiperazine as exemplified by **13** (L-775,606) which again showed >100 fold selectivity for the 5-HT1D receptor with sub nM potency. Oral bioavailability in rat, dog and monkey was ~25%.

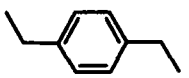


14 R = $NHSO_2Me$
15 R = $NHSO_2Ph$-4-NO_2

Investigation on the effect of chain extension of 5-O-alkyltryptamines showed that 5-HT1A receptor affinity reduced more rapidly than 5-HT1B affinity, with maximum potency (Ki 1nM) and selectivity (31) with the 5-nonyloxy analogue (32). A series of anilide derivatives has also shown nM affinity at both the h-5-HT1B and h-5-HT1D receptors (33) which is an extension of earlier work on a series of piperazides (34). The high potency and efficacy of examples such as **14** and **15** further confirms the finding that large substituents can be accommodated on the 5-position of the indole. This ability to tolerate large 5-substituents is further exemplified by the high potency and efficacy of a series of dimeric tryptamines with a diverse set of 5-substituent linkers, e.g. **16**, **17** and **18** which the authors claim supports the hypothesis for the presence of two 5-HT pharmacophores in the same molecule (35).



16 X =



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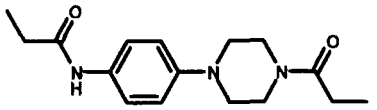

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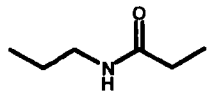
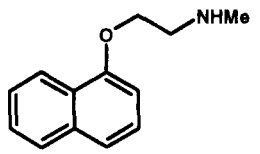
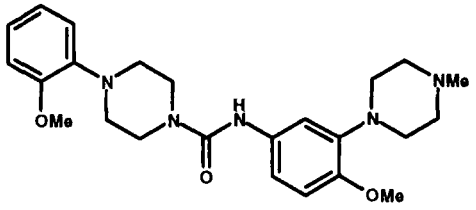


As an alternative to tryptamines, a series of naphthyloxyethylamines derived from propranolol have shown moderate to good affinity and efficacy, the best compound being **19** with both h-5-HT1B (Ki 26 nM) and h-5-HT1D (Ki 34 nM) affinity (36).



19

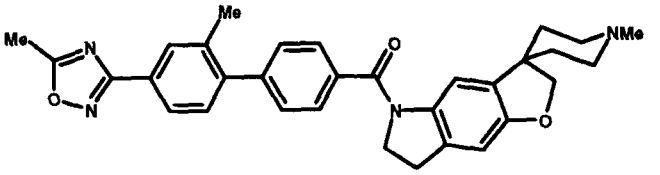
A model of the agonist binding site of the human 5-HT1A, 1B and 1D receptors has been published based upon the bacteriorhodopsin template (37). Revision may be warranted in light of more recent electron cryo-microscopy data which shows a major difference in the relative position of the aspartate containing transmembrane helix III (38, 39).



20

It has been known for some time that 5-HT1B/D receptors mediate auto-inhibitory control over the release of 5-HT from presynaptic nerve terminals, which suggests that they play a role in central 5-HT neurotransmission (40,41). The sub-classification of pre-synaptic serotonin autoreceptors in the human cerebral cortex as 5-HT1B (42) suggests that 5-HT1B receptor antagonists could be fast-acting antidepressants since acute blockade of these receptors would evoke an immediate increase in terminal 5-HT release. It has also been suggested that cortical and hippocampal 5-HT1B receptors play a role in

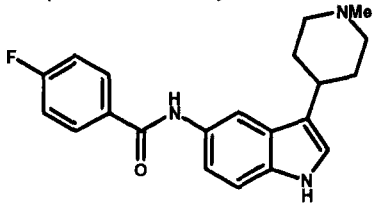
mechanisms of ethanol dependence (43,44). 5-HT1B/D receptor antagonists and their potential in depression have been reviewed (45,46) but recently, antagonists selective for the for the 5-HT1B receptor have been reported. The arylpiperazide, **20** showed 2 nM affinity for 5-HT1B receptors and was reported to be 45 and 350 fold selective over 5-HT1D and 5-HT1A receptors respectively (47). The spiropiperidine **21** (SB-224289) showed >80 fold selectivity for human 5-HT1B receptors over 19 other receptors and was an inverse agonist in the [35-S]-GTPγS binding model of 5-HT1B receptor function (48).



21

5-HT1E Receptors - Even though this receptor was identified some time ago (49), there has been no reported progress in the design of selective 5-HT1E ligands.

5-HT1F Receptors - The cloning of the human 5-HT1F receptor was reported in 1993 (50) and 5-HT1F receptor mRNA has been identified in rodent and primate brain regions (51). Autoradiography using non-selective ligands has been used to determine the distribution of 5-HT1F receptors in rat brain, where localisation was detected in cortical layers and caudate putamen (52, 53). It has been proposed that inhibition of trigeminal stimulation-induced neurogenic dural inflammation correlates with 5-HT1F receptor activation in the guinea-pig and that 5-HT1F agonists could find utility in the treatment of acute migraine (54). However it has also been reported that 5-HT1B/D receptors are the key mediators in migraine (55).



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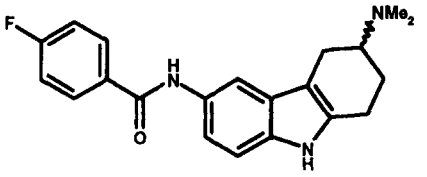
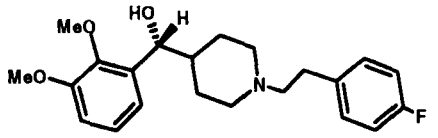


23

Compound **22** (LY334370) is a high affinity 5-HT1F agonist (Ki of 1.6 nM) and acts as a potent inhibitor of neurogenic dural inflammation in both rat and guinea-pig with estimated ID50 of 30 pg/kg p.o and 45 pg/kg p.o. respectively (56). Although **22** also has high affinity for the 5-HT1A receptor (11.9 nM) functional studies indicate that this compound has no 5-HT1A agonist or antagonist activity at doses that are several orders of magnitude higher than the dose that was fully effective in the rat dural extravasation model (57). More recently **23** (LY344864), has been shown to be a more selective 5-HT1F full agonist (Ki for 5-HT1F = 6 nM, for 5-HT1A = 530 nM). When **23** was administered orally 75 minutes before trigeminal stimulation in the rat neurogenic dural inflammation assay, an ID50 of 1.2 ng/kg was obtained implicating the involvement of the 5-HT1F receptor. However, the authors propose that further studies are necessary to fully characterise this response in this species (58).

5-HT2A receptors - Pre-clinical animal studies have provided good evidence for the role of 5-HT2A receptors in the pathophysiology and treatment of schizophrenia (59, 60), though many of these studies have used non-selective compounds which do not differentiate between 5-HT2 subtypes. A number of selective 2-HT2A antagonists are now available, the most investigated being **24** (MDL 100907) which has 5-HT2A

binding affinity of ~0.4 nM) and 100 fold selectivity over 5-HT2C receptors (61,62). In animal models, using reduction in amphetamine-induced hyperlocomotion as evidence of antipsychotic activity, **24** had an ED50 of 0.08 mg/kg i.p. with a much greater safety index when compared with a range of benchmark compounds. Imaging and occupancy of human 5-HT2A receptors has also been reported using [11-C]-MDL 100,907 and [123-I]-5-I-R91150 **25** as PET ligands (63-65).



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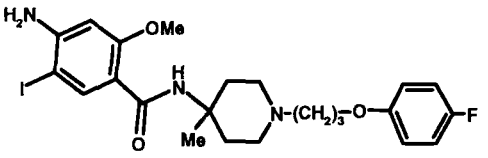
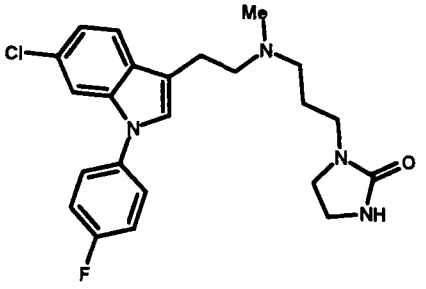


25

The different structural classes of both selective and non-selective 5-HT2A receptor antagonists has been comprehensively reviewed (60) and only the more recently reported selective compounds are described here. A series of ring-opened sertindole analogues furnished compounds typified by **26** which retained 5-HT2A receptor affinity but with improved selectivity over dopamine D2 and adrenergic α1 receptors (66). The authors also reported that these compounds had considerably lower affinity for 5-HT2C receptors, though no supporting data was given. The series of pyrimidino-piperazines has been extended to include additional examples from which the furan/thiophene combination **27** was the most selective for 5-HT2A receptors over 5-HT1A, though no data for 5-HT2C was included (67)



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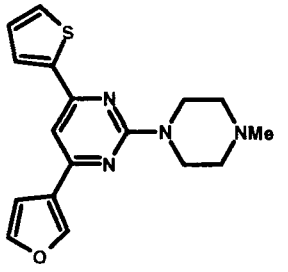
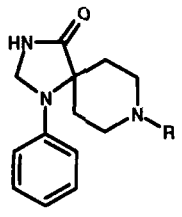


27

Spiperone **28** and **29** (AMI-193) have been reported to show ≥100 fold selectivity for 5-HT2A over 5-HT2B and 5-HT2C receptors (68). In the same study pyrido[4,3-b]indole and indoline analogues showed some selectivity, up to 10-60 fold with **30** but with reduced affinity (Ki 11 nM at 5-HT2A).



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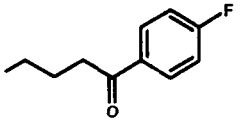

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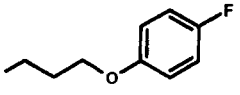
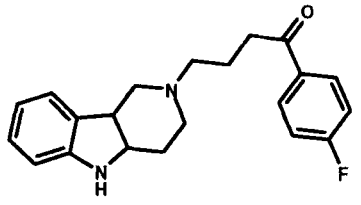
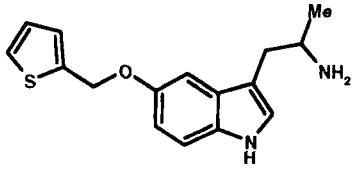




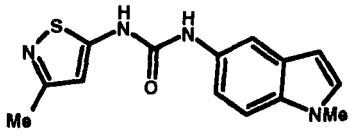
30

5-HT2B receptors - The human 5-HT2B receptor is the species homologue of the rat fundus receptor and has been implicated in the early steps of the generation of migraine headache (69). No truly selective agonists are known, but **31** (BW 723C86) was used to identify 5-HT2B receptor mediated anxiolytic-like activity



31

in the rat social interaction test (70) and hyperphagia and reduced grooming in rats (71). Compound **32** (SB-204741) has been described as a selective 5-HT2B receptor antagonist with pA2 of 7.8 in rat stomach fundus) (72). More potent, selective compounds have been identified from a series of tetrahydro-β-carbolines (73), typified by **33** (LY287375), which has pKB of 10.1.



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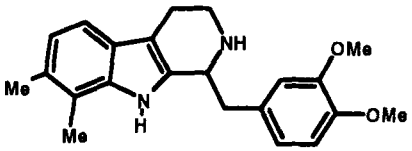
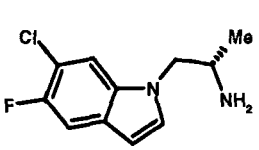


33

5-HT2C receptors - The 5-HT2C literature is fascinating in that preclinical animal studies support both agonists (74) and antagonists (75) as potential anxiolytics and antidepressants. Mice in which the 5-HT2C receptor gene has been inactivated have been reported to show obesity, epilepsy, cognitive deficit and a decrease in trait anxiety (76). A number of potent and selective agonists have been reported (77-81) including **34** (Ro 60-0175), **35** (Ro 60-0213, Org 35032), **36** (Org 37684), pyrazinoindoles (e.g. **37**) and **38**, Org 8484. Of interest, the *in vivo* pharmacodynamic model was penile erection in rats, an activity reportedly not seen in man (82). The agonists are active in a number of models of depression (pharmaco-EEG, restoration of bulbectomy-induced passive avoidance deficit, reversal of chronic mild stress-induced self stimulation reduction and differential reinforcement of low rates schedule) and also in anxiety and anxiety-related models (inhibition of defensive burying, inhibition of aversive brain stimulation escape, schedule-induced polydipsia paradigm and reduction of excessive scratching).



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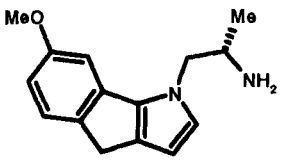

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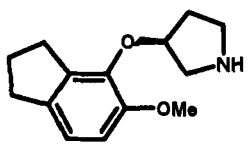

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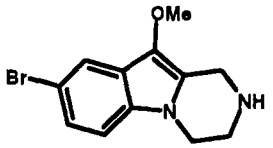

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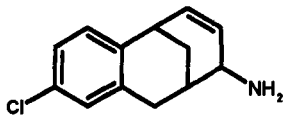
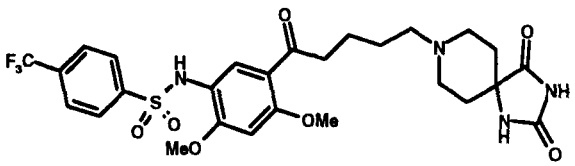


38

Potent and selective 5-HT2C receptor antagonists fall within two structural classes exemplified by the spiro-hydantoin **39** (RS-102221) (83), and the ureas **40** (SB-206553) (84,85) and **41** (SB-242084) (86). Compound **39** is a highly polar molecule and reported to have limited access to the brain (87). In contrast, the ureas show activity in the centrally-mediated pharmaco-dynamic



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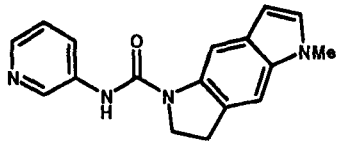

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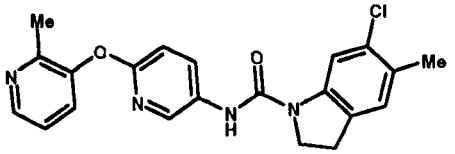
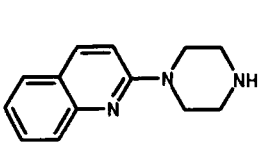


41

model of the reversal of m-chlorophenyl-piperazine-induced hypolocomotion and the anxiety models of social interaction and Geller-Seifter conflict tests (88).

5-HT3 receptors - The only proven indication for 5-HT3 receptor antagonists is in the treatment of radiation- and chemotherapy-induced emesis and in spite of the many proposals for the therapeutic application of 5-HT3 receptor antagonists for CNS disorders, none have been confirmed in the clinic (89, 90). The clinical effects of 5-HT3 agonists have yet to be explored. Pre-clinical studies have suggested a role in the modulation of acetycholine release (91) and potential for the treatment of anxiety (92), although emesis may be a problem with this class of compound (93). Quipazine **42** has been the lead compound (pKi 9.4) for the main structural class of 5-HT3 receptor agonists and partial agonists, with **43** (S 21007) being the most characterised (92) with a pKi of 8.85 and an EC50 of ~10 nM for stimulating the uptake of [14-C]guanidinium in substance P-stimulated NG 108-15 cells (93). Both [3,2-e] and [2,3-e]-fused thiophenes show high 5-HT3 receptor affinity (92) which can be further improved with a fused phenyl and in particular pyridyl **44**, pKi 12 and *in vivo* efficacy being dependent upon the aromatic substitution (94,95). A second series of 5-HT3 agonists was developed from 1-phenylbiguanide, for which a 70-fold increase in receptor affinity was achieved by 3-Cl substitution, a 100-fold increase for the 2-naphthyl and only a 2-fold drop in affinity for 3-chloro-phenylguanidine (96).



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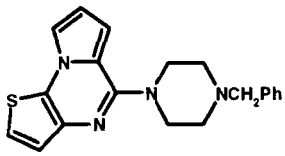

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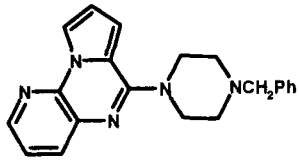
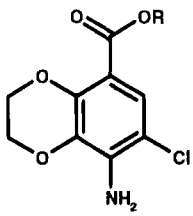


44

5-HT4 Receptors -The role of the 5-HT4 receptor in CNS function has been reviewed and its distinct localisation coupled with neurochemical and electrophysiological data suggest an involvement in cognition and anxiety (97-99). There is continuing interest from pre-clinical animal models in the potential use of 5-HT4 receptor agonists for memory enhancement (100, 101) and in the regulation of dopamine function (102). 25 years after their first description, benzamides are still being explored as 5-HT4 receptor agonists, although this is mainly for their potential as modulators of gastrointestinal motility (103-107).

There have been few developments in the field of 5-HT4 receptor antagonists since the review published last year (98), although **45** (SB-204070) and **46** (SB-207266) were recently reported to show modest anxiolytic activity in the rat social interaction model (108). Early clinical data are beginning to appear for **46** (109,110) but this is regarding its potential for use in irritable bowel syndrome. Clinical support for potential in CNS disorders is still awaited with interest for this class of drug.



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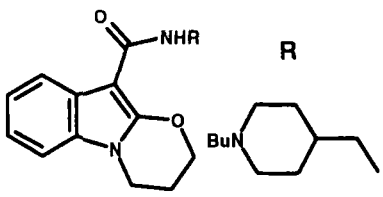

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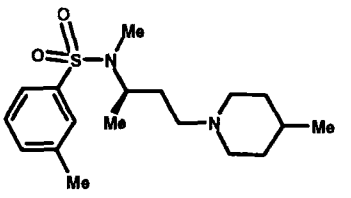
5-HT5 Receptors -The 5-HT5A and 5-HT5B receptors were recently identified by cDNA cloning. Mouse, rat and human receptors have been identified (111-115). When expressed in CHO cells the mouse 5-HT5 receptors exhibit similar pharmacological

profiles to 5-HT1B/D receptors (111). Roles for 5-HT5 receptors in brain development and regulation of astrocyte-mediated CNS pathologies have been suggested (113). Selective ligands for 5-HT5 receptors have yet to be identified but preliminary studies suggest that 7-hydroxy and 7-methoxy-1-naphthylpiperazines could be useful templates for the design of such ligands since they displace [3-H]-LSD from mouse 5-HT5A receptors with Ki values of 3 nM and 52 nM respectively (116).

5-HT6 receptors - The 5-HT6 receptor is of interest because of its expression in several brain areas, in particular the caudate nucleus (117) and a possible link between reduced extrapyramidal side effects of certain atypical antipsychotics (118). Centrally mediated function has been demonstrated in the rat using antisense to induce behavioural syndromes of yawning, stretching and chewing (119). Site directed mutagenesis has defined the agonist binding site (120). However, selective agonists and antagonists are eagerly awaited to help define the therapeutic potential of these receptors.

5-HT7 receptors - The molecular biology, pharmacology, distribution and function of the 5-HT7 receptor has recently been reviewed (121). Animal studies suggest that the receptor is located presynaptically in the CA1/CA2 region of the hippocampus and is also located in the periphery, in particular the coronary artery and the colon. A correlation between 5-HT7 receptor affinity and ability to protect against audiogenic seizures has been noted (122). The first selective 5-HT7 receptor antagonist, 47 (SB-258719) has recently been reported (123).



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Conclusion- The serotonin area persists in being the focus of intensive research since specific 5-HT receptor modulation offers opportunities for therapeutic intervention in a number of disease areas. Enormous progress has been made over the past decade in the design of selective ligands and these are helping to establish a clearer definition of the functional relevance of 5-HT receptors. Hitherto, clinical data have been derived mostly from serotonergic agents with mixed profiles but progression of more selective agents to the clinic will help clarify the role of this neurotransmitter, particularly in mental illness.

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Chapter 4. Recent Advances in Histamine H_3 Receptor Agents

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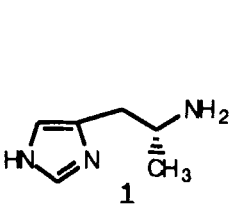
Introduction - The discovery of a third type of histamine (HA) receptor (1), the H_3 receptor, and the development of the H_3 subtype selective agonist, *R*-α–methylhistamine **1**, and antagonist, thioperamide, have stimulated a renewed interest in exploring the role of histamine as a neurotransmitter (2). The histamine H_3 receptor was originally characterized as a presynaptic autoreceptor that has been shown in rat brain cortex to control the synthesis and release of histamine (3). Furthermore, evidence has since been accumulated regarding the co-localization of modulating H_3-heteroreceptors on serotoninergic (4), cholinergic (5), noradrenergic (6), dopaminergic (7), and peptidergic (8) neurons. The H_3 receptor is most abundantly distributed in the CNS, with much lower levels present in peripheral tissues. However, the detection of histamine H_3 heteroreceptors on isolated enterochromaffin-like cells of rat stomach (9), and on lung (10), and cardiac tissues (11), has brought consideration of the H_3 receptor as an important general neuroregulatory mechanism for various physiological processes, not only in the CNS, but in peripheral tissues as well. Most importantly, recent medicinal chemistry efforts, which have been summarized (12-14), and pharmacology advances made towards the development of selective and potent H_3 receptor agents, have now provided opportunites for clearer elucidation of the role of this receptor subtype in human physiology and pathophysiology.

DESIGN OF HISTAMINE H_3 PHARMACOPHORES

Histamine H_3 Receptor Agonists - Several potent and selective H_3 receptor agonists have been reported (15). Compound **1** (K_i =1.5 ± 0.5 nM) was the first H_3 agonist introduced into clinical trials (2). In human volunteers **1** was well tolerated at dosages (p.o.) of several hundred mgs per day over several days (16). However, comparatively low plasma levels were attributed to extensive methylation of the drug's imidazole ring by histamine-*N*-methyltransferase as shown by the high recovery of tele-Me-*R*-α–methylhistamine in urine. To circumvent these metabolic issues, a series of azomethine prodrugs of **1** was designed (17). Compound **2** was selected for development (18). In humans receiving 0.1 mmol of **2** orally, plasma levels of *R*-α–methylhistamine-like immunoreactivity decayed with a $t_{1/2}$ of more than 24 h, the area under the curve being two orders of magnitude higher than after oral administration of **1**. Compound **2** exhibited anti-inflammatory and antinociceptive properties in rodents.



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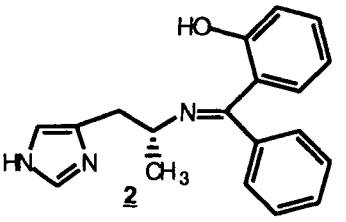

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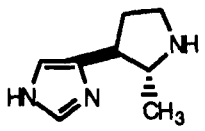
The H_3 agonist *R*-α–methylhistamine has been reported to elicit adverse bronchconstricter events by direct activation of H_1 receptors *in vivo* (ED_{50} = 1.7 mg/kg) which suggests this agent is less selective *in vivo* (19). Efforts directed towards the discovery of therapeutically useful H_3 receptor agonists devoid of undesired H_1 activity

0065-7743/98 $25.00

(20) have identified the cyclic, conformationally restricted pyrrolidine **3** (K_i = 2.8 ± 1.5 nM). Compound **3** has been shown to have a greater separation of H_3 and H_1 activities *in vivo* (H_3/H_1 ratio >> 550) than *R*-α–methylhistamine (H_3/H_1 ratio =17). No evidence of H_1 activity was detected at doses of **3** as high as 100 mg/kg, iv.

Even more potent in receptor binding studies (21) than *R*-α–methylhistamine is the H_3 agonist (α*R*,β*S*)- dimethylhistamine **4**. This compound (K_i = 0.8 ± 0.2 nM, EC_{50}= 3.4 ± 2.0 nM) displays a selectivity ratio of 130,000:1 for H_3 vs. H_1/H_2 receptors *in vitro*. However, **4** is also a weak substrate for histamine-*N*-methyltransferase. The K_m value of the enzyme for **4** is reported to be 2.8 μM and the V_{max}, 1.7 nmol/mg per h, these values being close to those reported for histamine (22).



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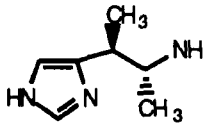

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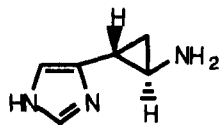

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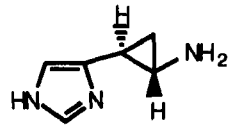
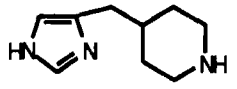


6

The stereoselective preparation of both enantiomers of *trans*-2-(1*H*-imidazol-4-yl)cyclopropylamine was recently disclosed (23). (1*R*, 2*R*)-*trans*-(1*H*-imidazol-4-yl)cyclopropylamine **5** (K_i = 3.5 ± 0.5 nM) was shown to have an order of magnitude better binding affinity for the H_3 receptor than its (1*S*, 2*S*) analog **6** (K_i = 23 ±1 nM). These studies accentuate the conformational preference of an anti-relationship between the basic side chain nitrogen and the 1*H*-imidazol-4-yl moiety for potent H_3 agonists. They also highlight the known enantioselective preferences of this receptor.

Potent and selective nonchiral histamine H_3 agonists have also been described. 4-(1*H*-imidazol-4-ylmethyl)piperdine **7** in binding studies in rat cortex with the H_3 antagonist [^{125}I]- iodophenpropit demonstrated two binding sites, K_H = 2.7 ± 0.5 nM and K_L = 1.01 ± 0.2 μM (24). This is in agreement with described displacement curves for other H_3 agonists. For comparison, **1** showed a K_H and K_L of 4.3 ± 3.4 nM and 0.22 ± 0.15 μM, respectively. Compound **8** (*S*-[2-(imidazol-4-yl)ethyl]isothiourea) is the most potent H_3 agonist reported to date, and has been shown to inhibit the binding of [^{3}H]-(*R*-α–methylhistamine) to rat brain membranes with a K_i = 0.1 ± 0.01 nM (25-26). The release of endogenously synthesized [^{3}H]-histamine induced by K^+-depolarization from rat brain slices and synaptosomes was inhibited by **8** with EC_{50} values of 1.0 ± 0.3 and 2.8 ± 0.7 nM, respectively. Compound **8** behaves as a full agonist and is about 4 times more potent than **1** and 60 times more potent than histamine. It was found not to be a substrate or an inhibitor of histamine-N-methyltransferase. After oral administration to mice, **8** decreased the tele-MeHA level in the cerebral cortex with ED_{50} values of 1.0 ± 0.3 mg/kg, an effect that was still maximal after 6 hr (26).



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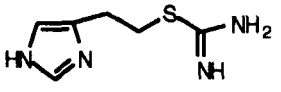

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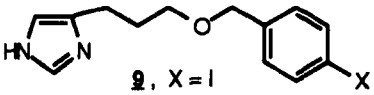


9, X = I
10, X = H

3-(1*H*-imidazol-4-yl)propyl(4-iodophenyl)methyl ether **9** was originally disclosed as a highly selective and potent H_3 receptor antagonist (27). This ligand exhibited a K_i = 5 ± 1 nM in a functional H_3 receptor binding study using rat brain synaptosomes. However, it was determined subsequently to elicit an agonist response in a selective

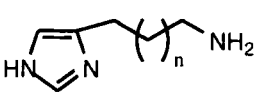
H_3 receptor guinea pig ileum bioassay (28). Both **9** and its desiodo derivative **10** were demonstrated to act as partial agonists at the functional H_3 receptor in mouse brain cortex, yet in guinea pig ileum, only **9** behaved as a partial agonist. In a related study, **9** and several 4-phenyl substituted analogs elicited agonist responses of varying magnitude that appeared related to the nature of the substituent in the 4-position of the aromatic ring (29). The data obtained from the bioassay, and conformational analysis (COSMIC) of these compounds (Table) led to the proposal that a gradual loss of agonism through the series was associated with an increased preference for adopting folded conformations. These folded conformations bring the imidazole and aromatic groups close together in space. It has been speculated that the role of histamine at the H_2 receptor is to mediate proton transfer across the receptor *via* its imidazole group (30). If the role of histamine at the H_3 receptor is similar to that proposed for the H_2 receptor, then the imidazole ring of these H_3 ligands most likely activates the H_3 receptor in the open conformation. Following this overture, agonist responses would be expected to be reduced as the majority of the conformations of these ligands become folded because π-stacking interaction between the imidazole and the remote aromatic ring would reduce the propensity for proton transfer (29).

X	% folded conformation	$pK_L \pm$ s.e.	% α[a]
H	100	7.7 ± 0.5	33
F	85	7.8	15
Cl	56	8.0 ± 0.3	58
Br	26	7.8 ± 0.3	84
I	33	8.2 ± 0.2	82

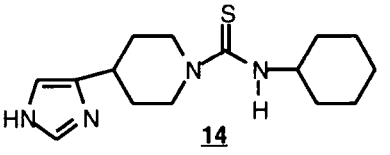
[a] Relative to *R*-α-methylhistamine; Table is adapted from ref 29

Table. Agonist Activity of 4-Substituted Phenyl Derivatives of **10**

Tissue dependent expression of agonist efficacy has also been observed for a number of homologs of histamine (31). A study of histamine homologs using two binding assays, [^{125}I]-iodophenpropit and N^{α}-[^{3}H]-methylhistamine binding to rat brain cortex membranes, and two functional H_3 receptor assays (inhibition of the neurogenic contraction in the guinea pig jejenum and [^{3}H]-noradrenaline release in mouse brain cortex slices), have provided some evidence for H_3 receptor heterogeneity. The histamine homologs **11**, **12**, and **13** all acted as competitive H_3 antagonists at the guinea pig jejenum. However, in the mouse brain cortex, they behaved as partial agonists. Compound **13** in the guinea pig jejenum, exhibited a $pA_2 = 8.4$; $pK_i = 9.1$ (N^{α}-[^{3}H]-methylhistamine binding), whereas it had a pD_2 of 8.2 ± 0.2 in mouse brain cortex ([^{3}H]-noradrenaline release). Additional studies are needed in order to determine if these observed discrepancies are due to differences in the efficiency of receptor coupling, tissue or species differences, or the existence of H_3 receptor subtypes.

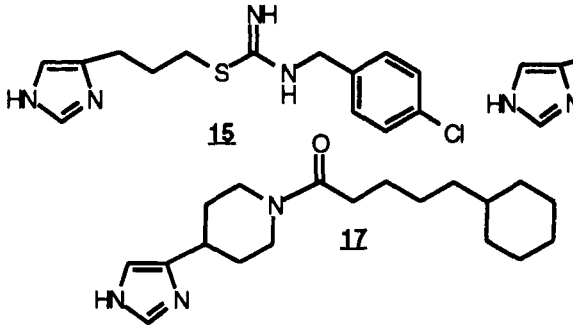


11, n = 1
12, n = 2
13, n = 3



14

Histamine H_3 Receptor Antagonists - Since the disclosure of thioperamide **14** (K_i = 4.3 ± 1.6 nM) as a potent and selective H_3 receptor antagonist (2), there has been a large number of 1*H*-imidazol-4-yl derivatives prepared and evaluated for their H_3 receptor affinity. The thiourea moiety of thioperamide was considered a liability for further drug development (32). Therefore, initial strategies employed in carrying out SAR studies for the development of selective and potent H_3 antagonists used 4-[1*H*-imidazol-4-yl]piperidine, histamine, histamine homologs, or **8** and its isothiourea homologs, as scaffolds, and investigated the utility of amide, thioamide, guanidine, urea, ester, and carbamate spacers as functional replacements for the thiourea moiety. These studies also investigated the optimal distance from the imidazole head group to the spacer, and the optimal distance from the spacer to an appropriate lipophilic tail group. Prominent H_3 antagonists (33-35) that emanated from these early efforts (33-38) were compounds **15** (K_i = 0.93 ± 0.06 nM), **16** (pK_B = 8.1), and **17** (K_i = 43.8 ± 3.0 nM). Compound **17** was shown to cross the blood-brain barrier and induced dose-dependent increases (10 and 30 mg/kg, i.p.) in histamine release at concentrations that exhibited significant histamine H_3 receptor occupancy (35).



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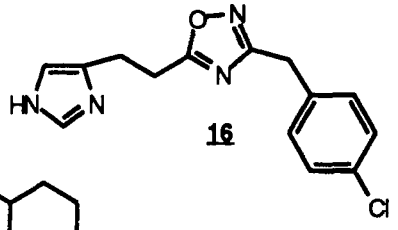

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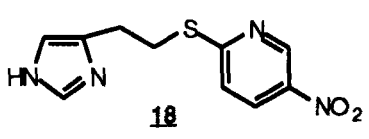
17

Advances in H_3 receptor pharmacology and the availability of several new radiolabeled H_3 antagonist ligands such as [^{125}I]-**9**, [^{125}I]-iodophenpropit, and [^{3}H]-*S*-methylthioperamide have intensified efforts directed towards selection of an H_3 antagonist clinical candidate (39-41). In this regard, several unique series of potent H_3 antagonists have recently been reported (42-43). Compounds **18** (K_i = 4.8 ± 0.9 nM) and **19** (K_i = 17 ± 3 nM) represent new antagonists that were discovered by using an approach for the preparation of brain penetrating compounds initially applied in the design of the H_2 antagonist, zolantidine (44). It had been demonstrated that amino-substituted aromatic nitrogen containing heterocycles could serve as functional equivalents of thiourea and urea spacer groups.



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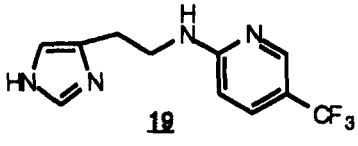
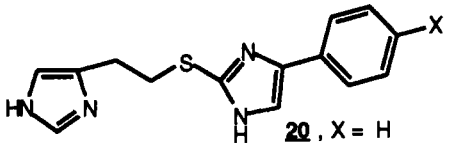


19

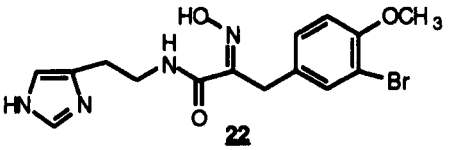
Access to the CNS can be impeded by several phenomena. Many of the selective and potent H_3 receptor antagonists reported possess functional groups, i.e. compound **15**, that can be protonated at physiological pH. Other distribution phenomena such as high binding to plasma proteins can preclude effective entry across the blood brain barrier. QSAR studies on a series of *para*- and *meta*-substituted 4(5)-phenyl-2-{[2-[4(5)-imidazolyl]ethyl]thio}imidazoles detailing octanol/water partition coefficients (log P), dissociation constants (pK_a), H_3 receptor affinity (pK_i), and H_3 antagonist potency

(pA_2) have been described (45). These studies yielded a QSAR model for this series that indicates that antagonist potency depends parabolically on lipophilicity and is decreased by bulky *para*-substituents. Compounds of this series are the thiolimidazoles **20** (pK_i = 7.85 ± 0.06) and **21** (pK_i = 8.53 ± 0.20).



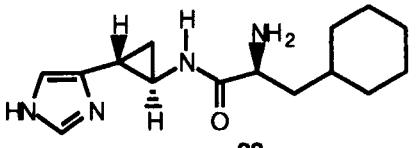
20, X = H

21, X = OSO_2Ph



22

Particularly interesting with respect to the search for and identification of new structurally entities as histamine H_3 receptor antagonists is the divulgence that verongamine **22**, isolated from the marine sponge, *Verongula gigantea*, exhibits selective H_3 receptor antagonist activity with an IC_{50} = 0.5 μM (46). Several new H_3 antagonists were developed by using **22** as a template for design (47-49). Structural modifications of **22** which led to the development of chiral H_3 antagonists included: (1) incorporation of a chiral *trans* cyclopropane ring for the 2-carbon straight chain between the imidazole head group and the amide-oxime spacer and/or (2) replacement of the amide-oxime moiety with olefin or allylic amine spacers. Important compounds identified from these studies were **23** (K_i =1.85 ± 0.5 nM), **24** (K_i = 1.0 ± 0.1 nM), **25** (K_i = 0.37 ± 0.2 nM), and **26** (K_i = 2.4 ± 0.2 nM).



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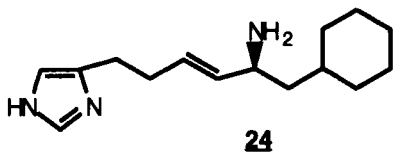

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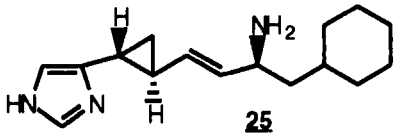

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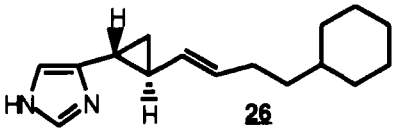

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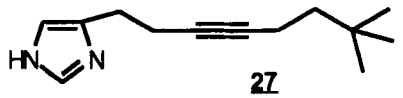

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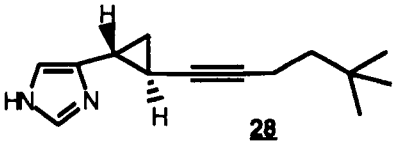

28

Following these efforts, potent and selective acetylene based series of H_3 antagonists were reported (50, 51). New antagonist compounds were obtained by several additional structural modifications which included: replacement of olefin or allylic amine spacers with an acetylene, and optimization of the hydrophobic tail feature using Topliss guidelines for aliphatic side chain substitution. Potent and selective H_3 antagonists identified from these studies were compounds **27** (K_i = 0.8 ± 0.04 nM) and **28** (K_i = 0.22 ± 0.1 nM). Importantly, these acetylene H_3 ligands with a planar, but non-polar spacer containing no heteroatoms have been demonstrated to cross the blood-brain barrier very efficiently, and elicit an antagonist response in guinea pig ileum functional studies (52, 53).

NEUROPHYSIOLOGY OF THE HISTAMINE H_3 RECEPTOR

The central nervous system (CNS) regional distribution of H_3 receptors in the brain parallels the areas known to receive histaminergic innervation (54, 55). A rostrocaudal gradient of H_3 cortical receptors is seen, with the frontal cortex receiving the highest density, and within the cortex, deep layers (IV-VI) having the greatest density (55). Limbic regions of the basal forebrain including the caudate nucleus, globus pallidus, olfactory tubercle and nucleus accumbens also contain high levels of H_3 receptors as well as the reticular part of the substantia nigra. The ontogenic development of the H_1 and H_3 receptors is different in both development as well as regional distribution patterns within the CNS, suggesting important yet separate functions (56). The abundance of H_3 receptors in several well-conserved limbic regions suggests an involvement in arousal, emotional, motor and cognitive functions.

Histamine containing neurons from the posterior hypothalamus have been well characterized and resemble the other biogenic amine systems in their topographically diverse terminal fields. Neuronally released histamine is known to be under tonic circadian control and influence many CNS homeostatic processes including arousal, body temperature, eating and drinking, attentional, learning and memory processes, neuroendocrine, locomotor as well as other activities (57). Histamine H_3 receptors have been identified on these histaminergic nerve terminals in the brains of rats as well as humans (1-2, 58-62). As described, this H_3 autoreceptor provides for a histamine receptor subtype that is uniquely positioned to regulate the amount of neuronally synthesized and released histamine and influence overall histamine tone in the CNS (62,63). The presence of H_3 receptors on non-histaminergic nerve terminals (i.e. heteroreceptors) has also been established by several labs (64-67).

Receptor distribution studies have demonstrated that the H_3 receptor is found in the highest levels in the brain with very low levels expressed in the periphery (68). However, H_3 receptors have been identified on parasympathetic and sympathetic nerve terminals, and peripheral functional assays have been used to determine H_3 agonist or antagonist properties. Typically, the H_3 receptor modulation of acetylcholine and/or norepinephrine release in the GI and cardiovascular systems is studied. Compound **1** has been shown to inhibit gastric acid secretion which suggests that it may have protective benefit against known ulcer-inducing agents (69). Inhibitory effects on neuropeptide release *via* H_3 receptor activation on sensory C-fibers has been described (18). Pharmacological differences have been seen between peripheral and CNS bioassays and could illustrate species, regional, or receptor subtype differences. Peripheral H_3 receptors may be functionally quiescent given a low histamine tone under normal physiological conditions. However, activation of these peripheral H_3 receptors provides modulatory effects that could be important under certain pathophysiological conditions.

THERAPEUTIC APPLICATIONS FOR HISTAMINE H_3 LIGANDS

Histamine H_3 Agonists - Numerous lines of evidence support a role of histamine and for H_3 receptors in arousal/vigilance or sleep/wake mechanisms (70-71). Recent understanding of the role of histamine in sleep/wake mechanisms have been gained

through the use of selective H_3 receptor ligands (71-73). Oral administration of **1** caused a significant increase in deep slow wave sleep in the cat (72) and rat (73) consistent with a reduction in histamine release and diminution in histaminergic tone. These findings suggest that histaminergic tone actively modulates whole brain activity and regulates arousal states and sleep/wake cycles in the CNS. It has been reported that azomethine prodrugs of **1** can be effectively used to liberate **1** *in vivo* at a sufficient rate (74). This approach highly increases the GI absorption and CNS penetration of **1**. Some of the newer pyrrolidine H_3 receptor selective agonists (75) appear to have better CNS penetration than **1**. Compound **3** (10 mg/kg, po) potentiated the pentobarbital-induced loss in righting reflex, increased total sleep time, promoted EEG activity consistent with physiological sleep (76).

The potential use of H_3 agonists for the treatment of migraine has been suggested (8,18). Activation of H_3 receptors on C-fiber sensory nerve endings inhibit the release of neuropeptides such as substance P and neurokinin. These vasoactive neuropeptides can cause vasodilation and increase vascular permeability in the blood vessels of the dura matter resulting in neurogenic inflamation and pain. H_3 agonists like **3** (10 mg/kg, po) have been shown to effectively inhibit the activation of neurogenic inflammation in a rodent model of migraine (76).

Sympathetic nerve endings (SNE) in the guinea pig (77), dog (78), and human heart (11) harbor the histamine H_3 receptor, whose function is to downregulate exocytotic norepinephrine (NE) release. In protracted myocardial ischemia, NE is also released by a non-exocytotic, "carrier-mediated" mechanism (i.e., by reversal of neuronal uptake), a process most recently substantiated in ischemic human myocardium (79). H_3 receptors are normally quiescent, but become activated in myocardial ischemia, when histamine is copiously released from mast cells (80). Recent studies reveal that H_3 receptor activation inhibits both neuronal and non-exocytotic, "carrier-mediated" NE release (81). Enhanced NE activity is a recognized cause of cardiac dysfunction and arrhythmias in myocardial ischemia (82) and hypertension (83), and these findings identify a new protective role for H_3 receptors.

Histamine H_3 Antagonists - Thioperamide **14** enhances arousal/vigilant patterns in a dose-dependent fashion in the cat (72) and in the rat (73). This has been also confirmed for newer non-thiourea H_3 blockers **17** and provides for a unique approach to several sleep disorders characterized by excessive daytime sleep (i.e. narcolepsy) or dysfunctions in HA tone and/or disruptions in circadian sleep patterns.

To date, several lines of evidence strongly suggest a role of neuronal histamine in cognitive processes (84-88). More recently, the use of H_3 antagonists in learning and memory disorders has been suggested (89, 90). It has been reported that two selective H_3 antagonists, **14** and **15**, produced significant improvements in a delayed non-matching to position task in rats, a model of short term memory (91). Thioperamide was also shown to improve learning and memory in a senescence-accelerated genetic mouse strain in a step-through (PAR) passive avoidance response (89). Thioperamide increased histidine decarboxylase (HDC) activity and improved response latency. In those studies, the senescence-accelerated control mice had reduced forebrain levels of HDC activity suggesting that improvement in HA neuronal activity could be useful in

age-related memory decline. Moreover, stimulation of H_3 receptors with 1 and 8 has been shown to decrease acetylcholine (ACh) from the frontal cortex and impair cognitive performance in both object recognition and PAR (90). In contrast, 1 was shown to improve recall in a water maze, suggesting opposite effects in a hippocampal-driven spatial learning paradigm (92). These findings might suggest differential influences by histamine H_3 receptor activation or blockade in either short term memory paradigms versus spatial learning tasks.

The ability of the histamine system to modulate neurotransmitter release and improve vigilant or attentional processes would further suggest the utility of H_3 therapeutics in ADHD and other attentional disorders. PET studies in ADHD children have indicated an asymmetry in the prefrontal cortex and caudate regions of the brain consistent with the attentional deficits and hyperactivity experienced by the patients (93-96). The presence of high levels of H_3 receptors in these regions suggest involvement in both attentional and motor systems. Studies have demonstrated improvements in acquisition in rat pup models with H_3 antagonists (97).

Histamine's role in eating and drinking homeostasis, body temperature, and hypothalamic hormone release suggest additional potential areas for therapeutic intervention (98). Chronic clinical use of H_1 blockers has been known to produce side effects such as weight gain. In contrast, direct icv injections of histamine into hypothalamic appetite centers attentuates feeding in rats. Dysfunction in histaminergic transmission has now been reported in genetically obese Zucker rats with dramatic reductions seen in both hypothalamic histamine levels and HDC enzyme activity (99). Moreover, H_3 receptors appear to modulate histamine release in critical eating centers in the hypothalamus.

Clinical evidence has clearly established a role of histamine in seizure-formation. Treatment of epileptic patients or overdosing with CNS-penetrating H_1 blockers in children can lead to clincal presentation of seizures. Recent animal data indicate that H_3 receptor antagonists decrease the incidence and frequency of electrically-induced seizures, thus suggesting a new approach to epilepsy (100-101).

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Chapter 5. Galanin Receptors: Recent Developments and Potential Use as Therapeutic Targets

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Introduction - The neuropeptide galanin has a widespread distribution in the central and peripheral nervous system of vertebrate and invertebrate species and has a broad range of biological actions which suggest potential therapeutic applications of galaninergic ligands in man. The effects of galanin include modulation of pituitary hormone secretion, inhibition of release of neurotransmitters that may play a role in memory acquisition or contribute to anoxic damage in the brain, modulation of appetite and sexual behavior, as well as effects on pain, gastrointestinal motility, heart rate and blood pressure (reviewed in 1). Human galanin is a 30 amino acid non-amidated peptide while in other species for which the entire sequence is known, it is comprised of 29 amino acids and is C-terminally amidated (2-6, see 7). Amino acids 1-14 are conserved in all species examined to date, except for a single substitution in tuna fish galanin (Figure 1).

Endogenous Galanin Peptides

Human	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS
Pig	----------ID-----H--Y--A(amide)
Rat, Mouse	----------ID--------H---(amide)
Dog	----------ID-----HE-P---(amide)
Cow	----------LDS----Q--H--A(amide)
Sheep	----------ID-----H--H--A(amide)
Chicken, Quail	----------D-----N--H-F-(amide)
Alligator	----------ID-----NE-H-IA(amide)
Frog	----------ID-----N--H--A(amide)
Bowfin	----------D----LN--H--A(amide)
Dogfish	----------D---
Trout	----------GIDG--TL---H--A(amide)
Tuna fish	-----A--------GIDG--TLG--P--A(amide)

Chimeric Galanin Peptides

M15	GWTLNSAGYLLGPQQFFGLM(amide)
M32	----------RHYINLITRQRY(amide)
M35	----------PPGFSPFR(amide)
M40	----------PPALALA(amide)
C7	----------[*D*-R]PKPQQ[*D*-W]F[*D*-W]LL(amide)

Figure 1. Amino acid sequences of endogenously occurring galanin peptides and chimeric galanin peptides. Dashes represent identity with human galanin sequence. The sequence of dogfish galanin is known only for amino acid residues 1-20. In the chimeric galanin peptides, galanin(1-13) is linked to the following C-terminal residues: M15, also known as galantide, Substance P(5-11); M32, Neuropeptide Y(25-36); M35, Bradykinin(2-9); M40, PPALALA (synthetic sequence) and C7, Spantide (Substance P receptor antagonist).

The use of truncated forms and analogs of galanin peptide, and of chimeric galanin peptides (Figure 1) which act differentially as agonists or antagonists in different physiological assays, has provided considerable pharmacological evidence for existence of galanin receptor subtypes, which may mediate specific effects of the

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peptide in discrete tissues or organ systems (reviewed in 7-9). In this report we highlight recent advances in molecular cloning of galanin receptor subtypes, whose characterization enables correlation with existing pharmacological data and assessment of potential therapeutic applications of galaninergic compounds.

GALANIN RECEPTOR MOLECULAR BIOLOGY, PHARMACOLOGY AND DISTRIBUTION

Molecular cloning of the first galanin receptor, GALR1, was reported in 1994, and in the past year, cDNA clones encoding two novel galanin receptor subtypes, GALR2 and GALR3, have also been described.

Table 1. Pharmacology of Cloned Galanin Receptor Subtypes

Receptor Subtype	Peptide Potency[#]	G Protein Effector; Cellular Response	M15;M32; M35;M40; C7
mGALR1-348aa		$G\alpha_{i/o}$; ↓cAMP	
rGALR1-346aa	Gal* > Gal(1-16) >> Gal(2-29)	$G\alpha_{i/o}$; ↓cAMP	Agonist
hGALR1-349aa	>>> [*D*-Trp^2]Gal ≡ Gal(3-29)	$G\alpha_{i/o}$; ↓cAMP	Agonist
rGALR2-372aa	Gal ≡ Gal(2-29) > Gal(1-16) >	$G\alpha_{q/11}$; ↑PI, ↑Ca^{2+}	Agonist
hGALR2-387aa	[*D*-Trp^2]Gal >>> Gal(3-29)	$G\alpha_{i/o}$; ↓cAMP ↑AA**	
rGALR3-370aa	Gal > Gal(1-16) >>> Gal(3-29)	ND***	

[#], Listed rank order of potency is based on rGALR1-3 binding studies.
*, Gal, Galanin(1-29); **, AA, Arachidonic Acid; ***, ND, Not Determined.

GALR1 - The first high affinity galanin receptor, GALR1, was cloned from human Bowes melanoma cells (10). Analysis of the full-length GALR1 sequence predicts the presence of seven potential transmembrane (TM) domains, characteristic of members of the superfamily of G protein-coupled receptors (GPCRs). cDNA clones encoding species homologs of GALR1 have been isolated from rat Rin 14B insulinoma cells (11,12) and mouse brain (13). Human, rat and mouse GALR1 are highly conserved, with overall identities of ≥92%. Genomic structure has been reported for human and mouse GALR1, and in both cases GALR1 coding sequences are contained on three coding exons, with conserved intron/exon junctions (13,14).

The pharmacology of galanin binding to heterologously expressed human (10,15), rat (11,12,15) and mouse (13) GALR1 has been studied in stably and transiently transfected mammalian cells. Binding of [^{125}I]-galanin to cloned GALR1 is saturable, exhibiting high affinity (K_d = 0.02 to 0.3 nM). The first two N-terminal residues of galanin are required for high affinity binding to rat GALR1: removal of Gly^1 significantly reduces high affinity binding and removal and/or modification of Trp^2 results in complete loss of high affinity binding (Table 1). Chimeric galanin peptides M15, M32, M35, M40 and C7 (Figure 1) also exhibit very high affinity, in the low nM range, towards rat GALR1. The pharmacology of human GALR1 endogenously expressed in Bowes melanoma and CHP-12 neuroblastoma cells (15,16), is similar to cloned human and rat GALR1. Despite global similarities and absence of a wide range of GALR ligands, subtle pharmacological differences distinguish human and rat GALR1. For example, in [^{125}I]-galanin displacement binding assays, [*D*-Trp^2]galanin(1-29) exhibits low affinity for human GALR1 but is inactive at rat GALR1 (K_i = 306 nM versus K_i >1000 nM). At

heterologously expressed human and rat GALR1, galanin causes a concentration-dependent decrease in forskolin-stimulated cyclic AMP accumulation. This response is eliminated by treatment with pertussis toxin, consistent with coupling of GALR1 to inhibitory G proteins. These results agree with signal transduction studies done in native tissues as well as in galanin receptor-expressing cell lines, where galanin has been shown to inhibit cyclic AMP accumulation in rat brain (17), hypothalamic and entorhinal cortical (18) and hippocampal (19) membranes, as well as in rat pancreatic Rin m5F (20) and Rin 14B (21) cells and human Bowes melanoma cells (16). At both endogenously as well as heterologously expressed human and rat GALR1, the chimeric galanin peptides M15, M35, M40 and C7 behave as agonists and inhibit cyclic AMP accumulation (16,22-24).

Regional distribution of GALR1 mRNA in rat brain has been examined by *in situ* hybridization and shown to be widespread but discrete (11,12,25-28). Highest levels of expression are seen throughout the basal forebrain, including the hypothalamus, especially in the medial preoptic area, paraventricular and supraoptic nuclei, ventral hippocampus, and amygdala, especially in the bed nucleus of the accessory olfactory tract. Significant expression is also seen in rat brain stem and the dorsal horn of the spinal cord. This suggests that GALR1 may mediate many of galanin's central effects, including feeding, cognition, neuroendocrine functions and modulation of sensory processing. Peripheral expression of human and rodent GALR1 has been studied by Northern hybridization. However, the data are limited and contradictory, and both restricted and widespread expression patterns have been reported (11,13,15). By Northern blot analysis, human GALR1 mRNA expression is also detected in fetal brain and small intestine (10). Furthermore, mRNA encoding GALR1 has been isolated from human small intestinal mucosal epithelial cells and shown to be present in this cell type throughout the length of the alimentary canal (29).

GALR2 - In the past year, a second galanin receptor subtype, GALR2, has been cloned from rat (30-33) and cloning of the human homolog, which exhibits 85% identity with rat GALR2, has also been reported recently (34). Sequence comparisons reveal a low level of homology between GALR2 and GALR1, with overall amino acid identity being about 38%. GALR2 coding sequences are interrupted by a single intron, a genomic organization distinct from GALR1. These observations suggest that GALR1 and GALR2 are highly divergent and may have evolved independently. Pharmacology of heterologously expressed human and rat GALR2 (K_d = 0.13 to 0.97 nM) is similar to GALR1 (Table 1). Rat GALR2, however, can be pharmacologically distinguished from rat GALR1 based on its significantly higher affinity for galanin(2-29) and [*D*-Trp^2]galanin(1-29). These two galanin analogs, therefore, can be classified as selective peptides for rat GALR2 versus rat GALR1. Unlike GALR1, stably expressed rat GALR2 primarily couples to activation of phospholipase C, causing elevation of intracellular inositol phosphate and Ca^{2+} levels, although coupling to inhibition of forskolin-stimulated cyclic AMP accumulation in transiently transfected COS-1 cells has also been reported (31). Furthermore, signaling through rat GALR2 stimulates arachidonic acid hydrolysis. Galanin-mediated arachidonic acid and phosphatidylinositol hydrolysis has been reported in mudpuppy (35) and human small cell lung carcinoma cells (36), respectively. Similar to rat GALR1, chimeric galanin peptides M15, M32, M35, M40 and C7 behave as agonists at rat GALR2 to stimulate phosphatidylinositol and arachidonic acid hydrolysis (24,33).

The pattern of expression of rat GALR2 mRNA has been examined by multiple approaches, including Northern blot, ribonuclease protection and *in situ* hybridization analysis (30-33,37,38). Expression is found in rat brain, spinal cord, anterior pituitary and peripheral tissues, including kidney, testis, skeletal muscle, liver, heart, spleen,

lung, stomach, small and large intestine. In the brain, GALR2 mRNA is detected in hypothalamus, amygdala, cerebral cortex, cerebellum, thalamus and hippocampus. The regional distribution of GALR2 mRNA in the brain overlaps, yet is distinct from the distribution of GALR1 mRNA. The results of subtractive receptor autoradiographic localization studies using [^{125}I]-galanin and [*D*-Trp^2]galanin(1-29) for selective displacement at GALR2 also indicate a substantial overlap in the distribution of GALR1 and GALR2 in many brain regions (39). The overlapping distribution patterns of GALR1 and GALR2 in hypothalamus, amygdala, hippocampus and spinal cord indicate that GALR1 and/or GALR2 could mediate the central effects of galanin on feeding behavior, neuroendocrine functions, cognition and sensory modulation.

GALR3 - The cloning of a third galanin receptor subtype, GALR3, from rat hypothalamus has been reported recently (40). The isolated rat GALR3 cDNA encodes a 370 amino acid GPCR, which shares 36 and 54% overall amino acid identity with the rat GALR1 and rat GALR2, respectively. In transiently transfected COS-7 membranes, specific [^{125}I]-galanin binding to rat GALR3 is saturable, exhibiting high affinity (K_d = 0.55 nM). Despite the very low levels of receptor expression, preliminary displacement binding experiments using galanin fragments suggest that rat GALR3 may be pharmacologically distinguished from rat GALR1 by exhibiting higher affinity for galanin(2-29) and from rat GALR2 by showing lower affinity for galanin(1-19) as compared to galanin(1-16). Furthermore, similar to GALR1 and GALR2, galanin(3-29) is inactive at rat GALR3. Galanin-induced functional coupling for rat GALR3 has not been reported. Future studies to attain higher levels of expression of GALR3 in heterologous expression systems and demonstration of agonist-mediated functional coupling should assist in further definition of the pharmacology of this galanin receptor subtype. By Northern blot analysis, rat GALR3 mRNA is reported to be present in heart, testis and spleen. No expression was reported in brain, lung, kidney, liver or skeletal muscle.

Other Galanin Binding Sites and Functional Responses - The existence of additional galanin receptor subtypes with discrete pharmacological profiles is suggested by both radioligand binding and functional analyses. The use of [^{125}I]-galanin(1-15) as a radioligand in autoradiographic studies of rat brain has allowed the identification of specific, high-affinity binding sites for this ligand in the dorsal hippocampal formation, neocortex and neostriatum, where [^{125}I]-Tyr^{26}-galanin(1-29) binding is minimal or absent (41). The functional significance of galanin binding sites that preferentially interact with the truncated peptide galanin(1-15) is not clear and their relationship to galanin receptors that have been described in dorsal hippocampus (19) remains to be defined. The existence of an additional galanin receptor subtype, in smooth muscle cells of the gastrointestinal tract, is suggested by the equipotency of galanin(3-29), galanin(1-29) and galanin(1-15) to cause relaxation of dispersed guinea pig gastric smooth muscle cells, concomitant with an elevation of intracellular cyclic AMP levels (42). Furthermore, galanin receptors that are functionally responsive to the truncated peptide galanin(3-29) have also been described in dispersed rat pituitary cells (43). In contrast to the [^{125}I]-Tyr^{26}-galanin(1-29) binding sites that have been demonstrated on human pituitary tumors (44) and the galanin receptors occurring on guinea pig gastric smooth muscle cells (42) that are activated by galanin(3-29), the galanin receptors on dispersed rat pituitary cells do not bind [^{125}I]-Tyr^{26}-galanin(1-29). They are detectable only with [^{125}I Bolton Hunter]-galanin(1-29), and are unresponsive to galanin(1-15).

Considerable use has been made of chimeric galanin peptides in efforts to elucidate the biological role of galanin and to characterize galanin receptor subtypes mediating particular effects. These peptides act as antagonists and inhibit galanin action in a range of functional assays, including inhibition of evoked release of

acetylcholine in the hippocampus, hyperpolarization of locus coeruleus neurons, modulation of spinal cord excitability and stimulation of feeding (see 7). However, they exhibit agonist activity in other galanin functional assays. For example, M40 acts as a weak agonist in inhibition of insulin secretion from mouse pancreatic islets (45) and M15 and M35 act as full agonists in relaxation of dispersed guinea pig gastric smooth muscle cells (46). Further characterization of cloned receptors in heterologous expression systems, together with detailed localization studies, will be necessary to achieve a greater understanding of the mechanisms of action of chimeric galanin peptides and whether their biological activity reflects the existence of additional galanin receptor subtypes with discrete pharmacological properties.

Molecular Modeling - The mode of binding of galanin to GALR1 has been studied using a combination of site-directed mutagenesis and molecular modeling (47,48). A three-dimensional model of GALR1 has been proposed using the experimental coordinates of the TM helices of bacteriorhodopsin, a seven TM protein with no homology to GPCRs (49). The galanin peptide has been modeled on the basis of ^{1}H-NMR evidence for highly populated α-helical conformations of free galanin in aqueous solutions, at least in the N-terminal Thr^3-Leu^{11} region, as well as α-helix that could be induced in other solvents (50,51). Alanine scanning mutagenesis of galanin(1-16) suggests a major role for Trp^2, Asn^5 and Tyr^9 in galanin receptor recognition (see 8,52). The proposed major ligand-receptor interactions are between the N-terminus of galanin with Phe^{115} in TM III, Trp^2 with His^{264} and/or His^{267} in TM VI and Tyr^9 through aromatic interaction with Phe^{282} in extracellular loop 3. This model predicts that the C-terminus of the galanin peptide extends beyond the limits of the helical bundle.

Recently Baldwin and co-workers have achieved a positioning of the individual helices of bovine rhodopsin, based on an extended library of sequences of GPCRs (53), with electron cryo-microscopy maps of a frog rhodopsin (54). As rhodopsin is a GPCR, the details of helix positioning and differences from bacteriorhodopsin are important and may now allow for a reassessment of the existing models of GPCRs. Future site-directed mutagenesis studies of cloned galanin receptors to establish rhodopsin-based models will almost certainly advance our understanding of the structure of the galanin binding pocket.

GALANIN RECEPTORS AS THERAPEUTIC TARGETS

Obesity - Galanin and galanin binding sites are present in high concentrations in rat, as well as primate and human, hypothalamus, particularly in nuclei associated with the control of feeding behavior (55,56). Galanin stimulates feeding when administered centrally into the paraventricular nucleus (57,58) or amygdala (59-60) of satiated rats and has been reported to selectively increase fat intake when rats are given a three-macronutrient choice diet (57). The effect of galanin on fat intake may be dependent on experimental conditions since studies employing different feeding paradigms were unable to demonstrate an effect on preferential fat consumption by galanin (61,62). Intraparaventricular administration of chimeric galanin peptide M40, which inhibits galanin-induced feeding, has been reported to reduce spontaneous fat intake in the 3-choice macronutrient paradigm (63) but shows no effect on spontaneous food intake in a fat-chow choice paradigm in rats (61). Furthermore, repeated intracerebroventricular administration of galanin for 14 days did not increase total daily food intake nor induce significant weight gain (62). Currently, the role of endogenous galanin in the regulation of feeding behavior remains unclear. Co-expression of multiple galanin receptor subtypes in rat hypothalamus emphasizes the need for the development of subtype-selective galanin receptor antagonists in order to investigate the role of galanin in the

regulation of food intake, and to assess their therapeutic potential as anti-obesity agents.

Cognition - Galanin has been reported to modulate the activity of cholinergic neurons and may therefore play a role in memory processes. In the rat ventral hippocampus, an area of importance to spatial learning, galanin inhibits cholinergic neurotransmission both presynaptically, by inhibition of acetylcholine release (64), and postsynaptically, by inhibition of muscarinic acetylcholine receptor signaling (65). Administration of galanin into the lateral ventricles, hippocampus or medial septum of rats impairs performance in memory tasks, primarily impairing the acquisition, but not retrieval, of spatial memory (66-68). The inhibitory effect of galanin on working memory has been reported to be attenuated by administration of the chimeric peptides M15, M35 and M40 (69-71).

In dementing illnesses, such as Alzheimer's disease, Down's syndrome and in many patients with Parkinson's disease, there is degeneration of cholinergic neurons projecting from the basal forebrain to the cerebral cortex. Galaninergic fibers and terminals hypertrophy and hyperinnervate surviving cholinergic neurons of the nucleus basalis of Meynert and diagonal band (72,73), leading to the suggestion that the cognitive deficit of such neurodegenerative disorders may be exacerbated by inhibition of acetylcholine release by galanin. Consistent with this are observations of preservation of galanin receptors (74,75) and elevated galanin levels in the cerebral cortex of afflicted individuals (76). The possible contribution of galanin to the cognitive deficit of disorders involving cholinergic dysfunction suggests therapeutic potential for galanin receptor antagonists, whose use in combination with anticholinesterase or cholinergic agonists may provide a potential treatment strategy.

Pain - The localization of galanin-like immunoreactivity in primary afferent terminals and in interneurons of the superficial dorsal horn of the spinal cord (77), together with demonstration of galanin binding sites on dorsal horn neurons (78), suggests a role for galaninergic transmission in the modulation of sensory processing, and particularly in nociception. However, the reported effects of exogenously administered galanin on nociception are contradictory, with antinociceptive, hyperalgesic and pronociceptive effects seen in response to mechanical stimuli (79-81). Nevertheless, both galanin and the N-terminal fragment galanin(1-15) potentiate the analgesic effects of intrathecally-administered morphine (82,83), and the chimeric peptides M15 and M35 are efficacious in antagonising the antinociceptive effect of opioid and non-opioid drugs (84,85). This raises the possibility that spinal galanin receptor agonists which potentiate the analgesic actions of morphine may be clinically useful in the treatment of chronic pain (for a report on novel molecular approaches to analgesia, see chapter 2).

The inhibitory effect of galanin on the nociceptive spinal flexor reflex is stronger after sciatic nerve axotomy than in intact animals (86), as is the facilitation of this reflex by the chimeric peptide M35. These observations suggest that galanin may be an endogenous analgesic factor that is of particular importance after nerve injury (87). In addition, the application of antisense oligonucleotides to galanin to the proximal end of a transected sciatic nerve, or chronic infusion of the chimeric peptide M35 after axotomy, results in increased self-mutilation (autotomy) (88,89). As this behavior is thought to reflect the development of neuropathic pain arising from ongoing spontaneous neuronal discharges in injured peripheral nerves, a role for galanin in suppressing this behavior and in controlling the development of neuropathic pain may be inferred. Suprisingly, the autotomy response to nerve injury is abolished in homozygous galanin knockout mice (90,91). Detailed characterization of the galanin

knockout mouse, including investigation of potential developmental deficits, should resolve this apparent discrepancy, and establish the exact role of galanin in neuropathic pain.

Nerve injury and nerve regeneration - Plasticity in expression of neuropeptides in response to nerve injury is well-documented (92), and up-regulation of galanin expression has been observed after axotomy of sensory, motor and sympathetic neurons (see 1). However, the functional significance of galanin expression in response to nerve injury is not clear. Of particular interest is that the galanin knockout mouse exhibits significantly diminished capacity for functional regeneration of sensory neurons after peripheral nerve transection (90,91), which suggests that galanin may play a role in mediating or facilitating the regenerative process in injured sensory neurons and raises the possibility that a galanin agonist may be of use in such therapeutic applications.

Within discrete regions of the brain, galanin modulates the release of a range of neurotransmitters, including inhibition of glutamate release from hippocampal neurons in response to anoxia (93) and inhibition of the release of aspartate and glutamate in response to depolarization in the ventral hippocampus (94). Furthermore, blocking neuronal activity or an imbalance in excitatory and inhibitory neuronal inputs will trigger increased expression of galanin (95,96). Thus, the ability of galanin to inhibit the release of excitatory neurotransmitters may reflect involvement in neuroprotective mechanisms *in vivo*, which may be of relevance in strategies for treating acute brain trauma or hyperactivity.

Neuroendocrine Disorders - Galanin is expressed in the hypothalamic-pituitary axis and in peripheral neuroendocrine tissues such as the pancreas, and has been demonstrated to have potent and species-specific effects on the secretion of pituitary and glucoregulatory hormones (see 8,97,98). In humans, intravenous infusion of galanin potently stimulates the secretion of growth hormone (GH) (97,99). This effect of galanin suggests potential for development of galanin agonists as GH secretagogues that may have utility in either the diagnosis or treatment of GH insufficiency syndromes. A variety of immunological and pharmacological approaches have provided evidence that galanin stimulation of GH secretion is mediated by effects on the release of either GH-releasing hormone (GHRH) (100) or somatostatin (101,102), while intracerebroventricular administration of a specific galanin-antiserum in rats decreases GH pulse amplitude and increases GH pulse frequency, suggesting tonic modulation of both GHRH release and somatostatinergic tone by galanin (103). In addition to actions on hypothalamic neurons, galanin released into the hypophysial portal circulation could act at the anterior pituitary as a hypophysiotropic factor. While data pertaining to galanin stimulation of pituitary hormone secretion by direct action on the pituitary gland are contradictory (104-106), demonstration of expression of mRNA encoding GALR2 in the rat anterior pituitary (32) is consistent with a biological role for galanin in anterior pituitary cells. An autocrine role for anterior pituitary galanin in stimulating GH secretion is suggested by correlation of elevated levels of GH secretion with expression of galanin in somatotrophs cultured from both wild-type C57BL/6S mice and mice exhibiting somatic hyperplasia arising from a GHRH transgene (107). It should be noted that the expression of galanin within human and rat pituitary shows cell-specific differences (see 108), and it is not yet clear how these differences relate to pituitary function.

Paradoxically to galanin stimulation of GH secretion in normal human subjects, the infusion of galanin into acromegalic patients has been reported to inhibit GH secretion (109). A normal GH secretory response to galanin infusion is restored in a

significant number of patients following neurosurgical removal of GH-secreting adenomas, while galanin inhibition of GH secretion can be demonstrated *in vitro* in a number of surgically removed adenomas (110) A significant proportion of such adenomas do not exhibit inhibition of GH secretion in response to the somatostatin analog octreotride (111), which is currently in use as a non-surgical therapy for acromegaly. This raises the possibility that galanin receptor agonists may be useful therapeutic agents for the treatment of acromegalic patients who are unresponsive to octreotride.

FUTURE DIRECTIONS

To date, three galanin receptor subtypes have been cloned and pharmacological data suggest the existence of additional receptor subtypes. Localization studies indicate substantial overlap in the CNS and possibly peripheral expression of the cloned galanin receptors. The development of subtype-selective galanin receptor agonists and antagonists is essential to provide experimental tools that would allow delineation of the role of each receptor subtype in mediating the wide spectrum of galanin's biological activities, and to define therapeutic indications for galaninergic compounds. As peptides generally do not cross the blood-brain barrier, peptidic galanin receptor agonists and antagonists will probably be of limited use for therapeutic CNS applications. The only small molecule galanin receptor ligand reported to date is a new spirocoumaranone related to the griseofulvin family of compounds, with a reported inhibitory activity (IC_{50} = 1.7 μM) at GALR1 (112). The development of selective high affinity small molecule galanin receptor ligands would allow realization and validation of their therapeutic potential as novel CNS medicines.

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Chapter 6. Sodium Channels: Recent Developments and Therapeutic Potential

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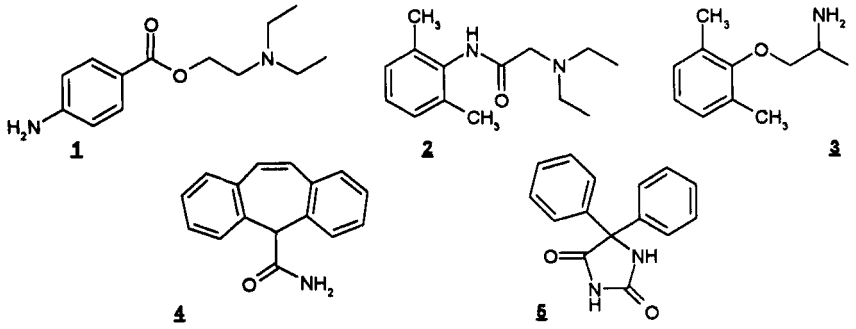
Introduction - It has long been recognised that the electrical excitability of cells is mediated by proteins which can modulate the ability of ions to travel across the cell membrane. Such proteins may be active transporters of ions, such as the sodium-calcium exchangers, or they may be proteins which (under certain, defined circumstances) allow the passive diffusion of ions down the concentration gradient which may exist across the cell membrane. These proteins are referred to as ion channels. Over the last ten years it has become increasingly clear that ion channels represent an excellent target for novel drug design (1). This report concerns recent developments in our understanding of the mechanisms which control the activity of a series of channels which are selective for sodium ions. In particular we will focus on the voltage regulated sodium channels which are found in the central nervous system, and emerging therapeutic applications of sodium channel modulators.

The family of sodium channels is a large, heterogeneous, group with members showing variations in mechanisms of regulation as well as variations in structure and tissue distribution (2). Genes encoding putative sodium channels have been isolated from numerous mammalian sources, including cardiac tissue, neural tissue and epithelial cells, and are also found in nematodes (*C. elegans*) and snails (*Helix aspersa*). These channels usually comprise a principle, ion conducting, α subunit in conjunction with two complementary, smaller subunits, usually referred to as β1 and β2. The α subunits of these channels are structurally diverse, arising from multiple sodium channel genes and alternative splicing events. Several families of sodium channel α subunits have been described, based principally on sequence homology. Different classes of sodium channels may have different complementary subunit requirements for full functionality, and in many cases the required complements to a particular α subunit have yet to be identified (3). It is now becoming clear that many drugs of a previously unknown mechanism of action actually act by modulating sodium channel conductance. These drugs include tertiary amine local anesthetics such as procaine (1), Class I antiarrhythmics such as lidocaine (2) and mexiletine (3), and anticonvulsants such as carbamazepine (4) and phenytoin (5), as discussed in Chapter 7 of this volume. It is through the use of drugs such as these that the role of sodium channels, and the potential importance of sodium channel blockers, in neuroprotection and analgesia has begun to be explored (4).



SODIUM CHANNEL HETEROGENEITY

Sodium channel proteins are notoriously difficult to purify, clone and express, and hence progress towards a full understanding of the role of individual channels has been slow. During

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the early days of this area of research the binding of neurotoxins such as tetrodotoxin (TTX) and saxitoxin (STX) were useful markers for sodium channel activity, with the eel electroplax providing the first sodium channel α subunit. Recent advances in molecular biology techniques have led to the identification of increasing numbers of distinct gene products in all areas, and sodium channel research has not escaped this. Currently nearly a dozen different mammalian sodium channel genes have been found, as shown in Table 1, and many more will doubtless follow. Splice variants occur and often have important functional consequences. Sequence homology is high between members of the group of channels found in the brain (around 75-88%) and these are assumed to have similar structures. If chemical similarity of the individual amino acids is taken into account the apparent homology between the brain sodium channels is actually much higher, suggesting a fairly recent divergence. The sodium channels found in peripheral sensory neurones, skeletal muscle and cardiac tissue show a lower homology (around 60%) to the brain channels. This difference between the channels is reflected in their differing sensitivity to tetrodotoxin, with the majority of peripheral channels being insensitive to this neurotoxin.

Gene Locus	Species	Other Names	Site of expression	Ref.
Scn1a	Rat	TypeI, NaCh1	Brain	(5)
Scn2a	Rat	TypeII, NaCh2	Brain	(6)
Scn3a	Rat	TypeIII, NaCh3	Brain	(7)
Scn4a	Mouse	SkM1	Skeletal muscle	(8)
Scn5a	Mouse	h1, SkM2	Heart	(9)
SCN6A	Human	HUMNACH	Heart, uterus	(10)
Scn7a	Mouse	Na-G	Glial cells	(11)
Scn8a	Mouse and Rat	NaCh6	Neuronal and glial cells	(12)
Scn9a	Rabbit	NaS	Glial cells	(13)
Unknown	Electric eel		Electroplax	(14)
Unknown	Rat	TTXi, SNS	Sensory neurones	(15)

Table 1: Cloned Sodium Channels

NEURONAL SODIUM CHANNELS

Structure - Native, neuronal voltage gated sodium channels exist as polypeptide multimers of an α subunit (260kDa) and subsidiary β1 and β2 subunits of 36 and 33kDa, respectively (16). Although the isolated α subunit demonstrates all of the basic pharmacology and physiology of a sodium channel, it has been shown that co-expression of α and β1 subunits modulates sodium channel characteristics (17). In addition it was recently shown that the β1 subunit may play a more important role *in vitro* than was previously thought, particularly in respect of the actions of neurotoxins and local anesthetics (18). The β2 subunit has been suggested to be involved in functional sodium channel expression and localisation (19). A single α subunit is thought to comprise an independent voltage dependent channel with four homologous domains (D1-D4), each with six transmembrane segments (S1-S6), as shown in Figure 1. Each transmembrane segment consists of an amphipathic α helix of about 22 amino acids. Although the three-dimensional structure is not known, it is generally assumed that the four sodium channel domains each fold similarly to models of voltage sensitive potassium channels (20), which consist of a tetramer of four identical, but separate, subunits. An extracellular pore-forming region between S5 and S6 forms a narrow selectivity filter containing several charged residues (21), and the entrance to the conductance pore is sited here. The S6 segments appear to line the channel pore, and it has been shown that this region is intimately involved with antiarrhythmic drug binding (22).

Voltage sensitivity - One of the transmembrane segments, the S4 in each of the domains, is highly charged, having multiple basic residues (4,5,6 and 8 in D1, D2, D3 and D4, respectively).

Membrane deploarization would be predicted to cause an outward movement of these segments with respect to the cell membrane, and it has been suggested that this forms the mechanism by which the channel senses voltage changes and converts transmembrane potential into conformational changes, resulting in a change in sodium ion conductance (23). Mutations of charged and hydrophobic residues within the S4 segments result in changes in the voltage dependence of the channel (24). More recent studies looking for the accessibility of cysteine residues to derivatisation with methanethiosulphonate reagents have confirmed that several residues in the S4 segment of domain D4 translocate from an intracellular to an extracellular position in response to depolarisation, and this may play a key role in both voltage dependence and gating of the channel (25).

Mechanism of Inactivation - Mutations or chimeras formed from specific regions of the primary amino acid sequence of sodium channels alter channel properties such as calcium and sodium selectivity. Site directed antibodies and site directed mutagenesis have been used to establish the residues forming the inactivation gate, which closes several milliseconds after channel opening (26). This intracellular loop is between D3 and D4 of the sodium channel α subunit and is 53 residues in length, with three amino acids in particular (isolecuine, phenylalanine and methionine, IFM, at positions 1488-1490) being crucial for inactivation. The IFM motif is suggested to act as the inactivation 'plug' for sodium channels, binding at the intracellular mouth of the sodium ion conductance pore (27). Inactivation also depends on residues in the intracellular loop connecting segments 4 and 5 in D4, which appear to form the binding site for the IFM motif (28). Quantitative analysis confirms that inactivation of sodium channels behaves much like the binding of a ligand (the inactivation loop) to a receptor, with binding affinity being a function of channel conformation (29).

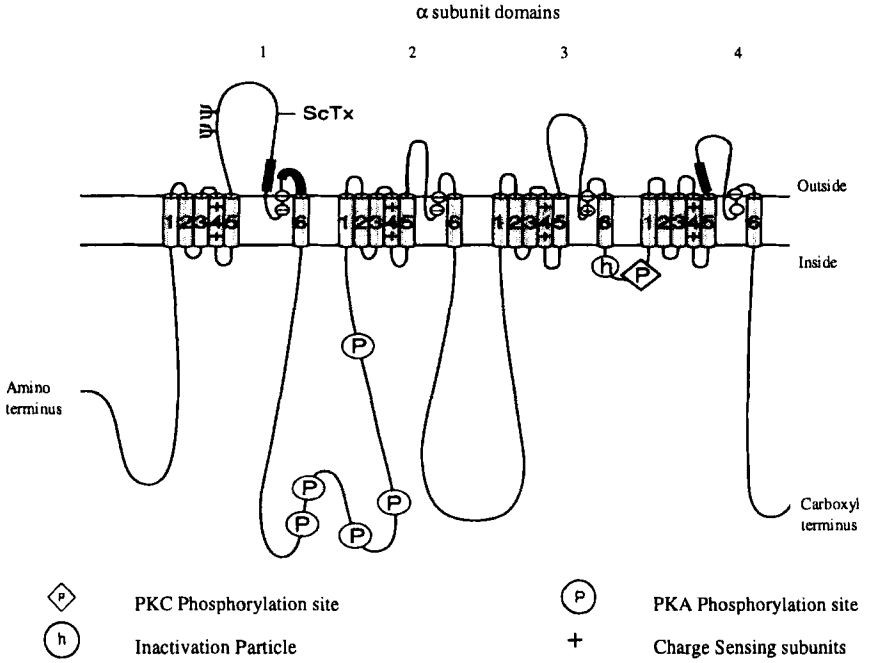


Figure 1: Sodium Channel α subunit structure

Function - The voltage-sensitive (or voltage gated) sodium channel is responsible for the increase in sodium permeability during the initial rising phase of the action potential in nerve (as well as in neuroendocrine, skeletal muscle and heart) cells. These electrically excitable cells maintain a high intracellular K^+ concentration and a low intracellular Na^+ concentration relative to the extracellular fluid. Hence the opening of sodium channels results in sodium entry into the cell, depolarisation, and therefore cell firing. Voltage clamp experiments reveal a biphasic nature to

sodium channel permeability; this has been attributed to an initial rising conductance (activation) followed by a period during which permeability returns to normal, resting, levels (inactivation). Thus three distinct sodium channel states can be observed: resting (or closed), activated (open) and inactivated (closed and blocked). Inactivation proceeds many thousands of times faster when channels are open compared to the resting state.

Drugs that interact with sodium channels to block ion flux cause the channels to inactivate to a greater extent and with smaller depolarisations than they would normally. The relatively slow off-rate of drugs such as the anticonvulsant phenytoin means that there is an accumulated block with repeated depolarisations (use-dependent block). This means that, in the case of therapeutically relevant concentrations of phenytoin (8 mM) channel block is only significant if cells remain depolarised for at least 5 seconds. This may explain why many drugs of this type do not alter normal action potentials or excitatory synaptic potentials - these events last less than 200 ms. Recent studies with the neuroprotective compound riluzole have shown similar results to those found with phenytoin. Riluzole was found to block cloned recombinant type IIa channels (in oocytes) only at high concentrations, whereas at low concentrations a preferential block of the inactivated state of the channel was observed (30). Thus sustained depolarisations, as occurs during ischemia or seizures, greatly enhances the blocking action of phenytoin and similar drugs. Hodgkin and Huxley first described the three-state model for sodium channels, and a theory to explain use-dependent blockade of ion channels, termed the 'modulated receptor theory' (MRT), has been developed based on their observations. This theory assumes that sodium channel blockers bind different channel states with different affinities, and that drug binding alters the transition rates between different states (31). The MRT states that:

- The three normal channel states are: *Resting (R), Open (O)* (or activated), and *Inactive (I)*
- Under normal resting conditions the channels are predominantly in the *Resting* state and are nonconducting
- When the membrane is depolarised, the channels *Open* and conduct sodium, resulting in the inward sodium current that makes the major contribution to the action potential
- The inward sodium current rapidly decays as channels move to the *Inactive* state
- The return of the *Inactive* state to the *Resting* state is termed reactivation and is voltage- and time-dependent
- Drug binding results in transitions to R^*, O^* and I^* channel states. These '*' states have different transition rates between them compared to the normal states

More complex models have been developed to explain the apparent ability of many sodium channel blockers to distinguish between normal conductance and the sustained burst of activity which occurs during, for example, an epileptic fit. These models propose the existence of two inactivated states, one of which arises from a brief depolarisation (the fast inactivated state), which is explained by the ball-and-chain model of channel inactivation, and a second, slowly inactivated, state which arises following sustained depolarisation. It has been proposed that the selectivity of compounds such as lamotrigine (7, *vide infra*) arises from a specific binding to the slowly inactivated state, stabilising the channel in this conformation and therefore making less channel available for activation (32). However, other studies suggest that the use-dependent action of these compounds could also be explained by the slow, cumulative, binding of this type of compound to the fast-inactivated state of the sodium channel (33).

REGULATION OF NEURONAL SODIUM CHANNELS

Phosphorylation - One of the means by which brain sodium channels have been found to be regulated is phosphorylation, and both protein kinase A and protein kinase C are involved. Phosphorylation by protein kinase C has been shown to reduce curent magnitude (34) and can also slow the rate of fast inactivation (35). The target for PKC appears to be a serine residue (S1506) in the linker between domains 3 and 4 (see Figure 1). The effect of phosphorylation by protein kinase A is to reduce sodium current amplitudes by reducing the open probability of the

channel, an effect which is only observed when S1506 has already been phosphorylated by PKC (36). Of the five PKA consensus sites in the linker between domains 1 and 2 of the type IIA sodium channel, four have been shown to be phosphorylated by PKA (37), and the same four sites are found to be dephosphorylated by phosphatase 2 and calcineurin (38). Moreover, functional effects of PKA activation were recently reported and confirmed to be mediated by phosphorylation of the PKA consensus sites in the linker region between domains 1 and 2 of the rat brain IIA alpha subunit (39), and work is underway to determine the precise role of potential phosphorylation sites in channel regulation (40).

Neurotoxin Binding - A wide range of toxins have been found to act on sodium channels to either block the channel or to interfere with the activation or inactivation of the channel. On the basis of structural studies and binding information it has been deduced that there are six different toxin binding sites. Toxins acting at Site 1 are associated with paralytic food poisoning and classically contain a positively charged guanidinium moiety, as in tetrodotoxin and saxitoxin. Sodium channels are permeable to simple guanidinium ions, and they are attracted to the mouth of the pore by the same mechanism which attracts sodium ions, but in the case of the bulky neurotoxins the pore becomes blocked by the rest of the molecule. Site directed mutagenesis experiments to replace the negatively charged glutamic acid moiety at residue 387 with glutamine results in a 1000-fold reduction in the sensitivity to this class of toxin. A model of the site of action of TTX and STX has recently emerged which takes into account the above observations and also incorporates information on the crystallographically determined structures of TTX and STX. This model confirms a crucial role for the extracellular segments between S5 and S6 in forming part of the lining of the pore and also in forming part of the binding site for the guanidinium toxins (41). The model accurately predicts the changes in sensitivity to these neurotoxins on the basis of different interactions with different sodium channel isoforms.

The lipid soluble toxins (binding site 2) appear to bind in the hydrophobic domain of the cellular membrane, as do the brevetoxins (sites 5), while binding sites 3 and 4 are on the extracellular surface of the α subunit. Labelling studies with the neurotoxin batrachotoxin (site 2) reveal a binding site near the transmembrane segment S6 of domain 1 on the α subunit of recombinant sodium channels (42). This site is adjacent to the determined binding site of brevetoxin (site 5), at a transmembrane region between domains 1 and 4 (43), implying a common area for binding of hydrophobic toxins. It is becoming increasingly clear that these neurotoxin binding sites do not exist in isolation to each other and examples of allosteric interactions between the binding sites are frequently discovered. Recent studies, for example, show a role for brevetoxin (site 5) in the modulation of scorpion α toxin binding (site 3) (44), with further studies showing that tetrodotoxin (site 1) reverses this effect (45). Such observations serve to emphasise the care needed in interpreting results from binding studies or guanidine flux experiments, which often incorporate neurotoxins in the protocols. It is clear, however, that studies on the interactions of these neurotoxin binding sites will soon begin to tell us how and where to best interfere with sodium channel activities for therapeutic benefit. The pyrethroid insecticides, analogues of the natural toxins found in the flowers of the chrysanthemum family, are well known for having effects on a variety of neuronal receptors but their primary cause of toxicity is due to binding and stabilisation of the open state of voltage gated sodium channels. The binding site for this class of compounds is referred to as site 6, and it has recently been shown, through photoaffinity labelling, that this site lies on the α subunit of the type IIa sodium channel (46).

Drug Binding - The site of action of antiarrhythmic, anticonvulsant and local anesthetic drugs is believed to be on the intracellular side of the sodium channel (47). Most studies on sodium channel mutants have been carried out with local anesthetic drugs, and it is found that single amino acid alterations at residues 1764 or 1771 do not alter channel gating but decrease local anesthetic action (48). Mutations at 1769 enhance local anesthetic block, while substitution also alters selectivity of molecules for permeation through the channel. The molecular site of local anesthetic block is thus concluded to be within the ion conductance pathway, near the

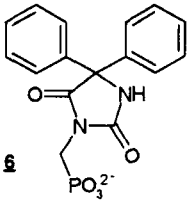
inactivation gate and the selectivity filter (49). It was suggested that the ionisable amino group binds to the selectivity filter of sodium channels, in the same way that tetraethylammonium ions block potassium channels, and mutation studies have provided clear evidence in support of this. More recent studies with phenytoin and lidocaine suggest an interaction of these drugs with the same site (50). This site appears to be neurotoxin binding site 2, with drugs binding to the site in a frequency and voltage dependent manner (51). However, the possibility of a binding site for sodium channel blockers which modulates the binding of ligands such as BTX to neurotoxin binding site 2 through an allosteric interaction cannot be ruled out (52). Selective high affinity binding of these inhibitors to the inactivated state of the channel is believed to be the property that makes them specific for the blockade of abnormally firing sodium channels without inhibiting normal cardiac and neuronal sodium channels. However neither lidocaine or phenytoin require sodium channels to cycle through the open state for use-dependent binding to occur; repetive depolarising impulses that are insufficient to cause channel opening still cause increased blockade with these agents (50).

THERAPEUTIC USE OF NEURONAL SODIUM CHANNEL BLOCKERS

Sodium channels have been suggested to play a role in (and sodium channel blockers to be useful in treating) many disorders of the central nervous system. The majority of the compounds studied to date show some potential in more than one of these disorders; very few compounds with selective anticonvulsant, analgesic or neuroprotective activity have been identified. However, it is possible that discrete sodium channels are involved in the pharmacology of each condition, giving the potential for selective compounds with a clearly defined usefulness. This point will only be clarified when a range of neuronal channels have been expressed in isolation and selective blockers identified.

The structural similarity between the different cation channels means that selectivity is a serious issue; many sodium channel blockers also have effects on calcium channels (mexiletine and lifarizine), while others effect potassium channels (lidocaine). Selectivity over peripheral sodium channels is also essential for any therapeutically useful neuronal sodium channel blocker. Successful neuronal sodium channel blockers will obviously have to address these problems as well as demonstrating a lack of effect on normal action potentials. In fact the majority of the newer generation of sodium channel blockers have been found to be 'use-dependent' - their activity increases in situations where rapid neuronal firing is sustained for several seconds. This means that the more potent compounds have little or no effect on normal neuronal activity.

Anticonvulsants - Neuronal sodium channel blockers found their first major application with the discovery of their potential as new treatments for epilepsy, and this area of therapy has seen a minor revolution with the arrival of more potent and selective compounds offering improved control over this debilitating condition (see Chapter 7 of this volume). New sodium channel blocking anticonvulsants that are now finding clinical use include fosphenytoin (**6**), lamotrigine (**7**) and zonisamide (**8**).



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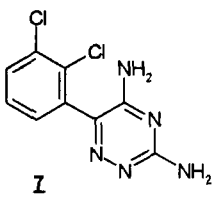

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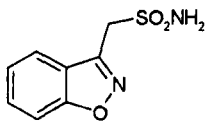

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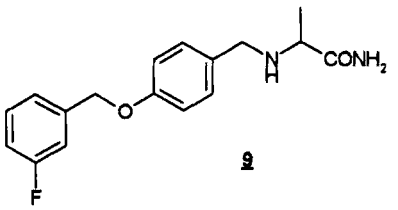
Evidence for the role of sodium channel block in the activity of the current group of new anticonvulsants is based on the similarity of their actions to those of TTX in rat hippocampal slices. Electrophysiological studies support selectivity of action of phenytoin and lamotrigine on persistent bursts of sodium channel openings, with little effect on normal action potential activity. Recently reported anticonvulsants include a series of propanamide derivatives, typified by PNU-151774E (**9**), and several 3-aminopyrroles, exemplified by AWD-140190 (**10**). These

compounds show potent activity in the maximal electroshock (MES) model for anticonvulsants, (ED_{50} of 8 mg/kg and 2.5mg/kg, respectively) and also show excellent selectivity over neurological impairment (a common side effect of many sodium channel blockers), measured in the rotorod assay (53,54).



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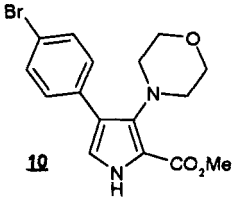

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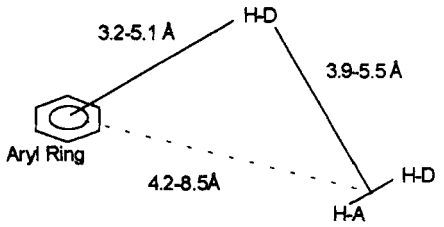
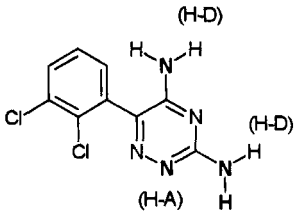
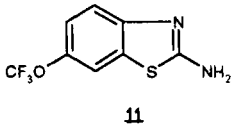
Consideration of the activity of the 3-aminopyrroles, in conjunction with the other known anticonvulsants such as lamotrigine, phenytoin, carbamazepine and zonisamide, led to the suggestion of a pharmacophore model of the interaction of these compounds with their sodium channel binding site (54). This pharmacophore incorporates an aromatic ring with a hydrogen bond donor (H-D) moiety and a separate hydrogen bond donor-acceptor (H-A and H-D) group, as illustrated for lamotrigine.





Neuroprotective Agents - Stroke and other brain injuries are major causes of mortality and morbidity in the adult population for which no efficient therapy is presently available (55). Despite considerable study, the mechanisms causing irreversible brain damage after ischemia are not fully understood and the primary event in this catastrophic cascade remains subject to conjecture. Nevertheless, the energy failure which results in cellular damage following head injury certainly involves changes in cellular ion homeostasis (56); anoxic neurones are rapidly depolarised and thus voltage sensitive sodium and calcium channels will be opened (57). Excitatory amino acids such as glutamate and aspartate are clearly implicated in the pathogenesis of neuronal death (58), and numerous studies have demonstrated that antagonists of glutamate release can attenuate ischemic brain damage in animal models of focal ischemia (59). Selective blockade of sodium channels with TTX slows or reduces many of the markers of ischemia-induced cell damage, and local anesthetics have been shown to have neuroprotective properties. Further studies have revealed the potential of many of the classical, and newer, anticonvulsants to protect against ischemia and there is now a substantial body of evidence to suggest that voltage-dependent sodium channel blockers are neuroprotective in models of global and focal ischemia (60,61,62).



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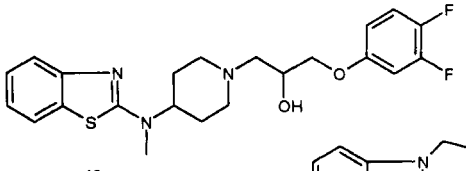

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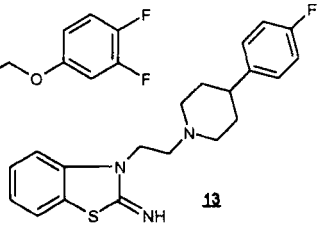

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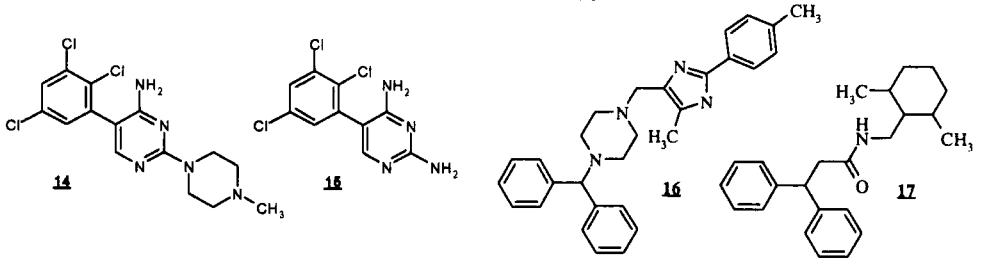
Sodium channel blockers act more effectively during conditions of cellular depolarization, sustained for seconds. This should result in the compounds having little effect on normal neuronal signalling, but allow the blockade of sodium channels during pathological conditions such as seizures or ischemia. Many of these agents (phenytoin, lidocaine, carbamazepine,

lamotrigine and others) are cerebroprotective at doses that are relatively free of side effects, in marked contrast to excitatory amino acid antagonists or L-type calcium channel blockers. One chemical series that has been particularly productive for the identification of neuroprotective sodium channel blockers is the benzothiazole system, represented by structures **11**, **12**, and **13**, as shown in Table 2.

Table 2. Activities of Selected sodium channel blockers in the Middle Cerebral Artery Occlusion (MCAO) model of neuroprotection.

Compound	Reduction in Infarct Volume	Ref.
Lubeluzole (**12**)	50% at 0.3mg/kg bolus then infusion	(63)
BW619C89 (**14**)	48% at 20mg/kg	(64)
BW1003C87 (**15**)	46% at 20mg/kg	(65)
Lamotrigine (**7**)	52% at 20mg/kg	(66)
Carbamazepine (**4**)	24% at 2x50mg/kg	(67)
Phenytoin (**5**)	40% at 2x100mg/kg	(67)
RP66055 (**13**)	44% at 2x8mg/kg	(67)

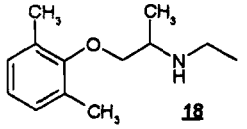
A further group of neuroprotective sodium channel blockers has been identified in derivatives and analogues of the antiarrhythmic drug lidocaine, **2**. These include the mixed sodium/calcium channel blocker lifarizine, **16**, which is widely found to be neuroprotective in models of global and focal ischemia (68), and a series of lipophilic amide analogues (such as PD85639, **17**) which are found to be neuroprotective in a hypoxia model (69).



A model of the membrane potential alterations which occur following an ischemic event, mimicked *in vitro* by treatment with veratridine, has been used to evaluate several sodium channel blockers and gives IC_{50} values for lubeluzole, riluzole, lamotrigine and phenytoin of 0.54, 1.95, 10 and 16.1, respectively (70). Several of these sodium channel blockers have also been studied in models of head trauma, such as the rat fluid percussion model. The necrosis which follows a blow to the head appears to develop as a result of an interruption in blood flow causing edema and ischemia. BW1003C87, **15**, and BW619C89, **14**, both reduce the damage observed following head trauma and it is likely that many other voltage dependent sodium channel blockers would also be protective in this model (71,72).

Analgesic Agents - Inappropriate impulse generation within injured axons and their corresponding dorsal root ganglion (DRG) neurones has been suggested to be the mechanism for formation of neuropathic pain following nerve injury, and this process has been shown to be dependent on sodium channels (73). Both lidocaine and carbamazepine have been shown to effect a use-dependent block of sodium channels in DRG neurones 18 days post-axotomy (74), and carbamazepine was recently shown to relieve paresthesias following damage to the ilioinguinal nerve during herniorraphy (75). Sodium channel blockers have also been shown to have local anesthetic activity (76), and several of the drugs already established as local anesthetics or anticonvulsants (carbamazepine, lidocaine, phenytoin, lamotrigine) have been found to be either useful in the treatment of neuropathic pain, such as from trigeminal neuralgia or diabetic neuropathy, or to be active in animal models of these disorders (77, 78, see also

Chapter 2 of this volume). The effects of these drugs are thought to be mediated centrally as local administration to the hyperalgesic paw is ineffective (79). In studies aimed at differentiating between analgesic activity (pain response in a normal paw) and hyperalgesic responses (pain responses after PGE_2 injection) lamotrigine clearly had no effect on normal pain responses but blocked completely the hyperalgesic pain response. The same study, using streptozocin-induced diabetic neuropathy, showed that intrathecal lamotrigine could block hyperalgesia for as long as two days (80). A mexilitine analogue, **18**, has recently been claimed to be particularly useful in the treatment of neuropathic pain (81).



18

Conclusions - Over the last few years several new classes of voltage-dependent sodium channel blockers have come to light, and these are being studied as new treatments for various, often presently poorly addressed, disease states. Our increasing awareness of the importance of these channels and their therapeutic usefulness will only serve to extend this trend and will drive research into the identification of new agents with ever more selective actions. Perhaps the most crucial step forward will be the development of expression systems for more recombinant channel proteins, allowing a more thorough and straightforward route to the identification of sodium channel blockers that are selective for individual channel subtypes.

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Chapter 7. Recent Progress In Antiepileptic Drug Research

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Introduction - Epilepsy is a relatively common neurological condition affecting 0.4-1% of the world's population (45-100 million people). For the general populace there are approximately 20-70 new cases per 100,000 diagnosed each year with a 3-5% lifetime probability of developing the disease (1,2). The older established antiepileptic drugs (AEDs) phenytoin, carbamazepine, clonazepam, ethosuximide, valproic acid and barbiturates, are widely prescribed but suffer from a range of side effects (3). Furthermore, there is a significant group of patients (20-30%) that is resistant to the currently available therapeutic agents. Since 1989 several new drugs have been launched, including felbamate, gabapentin, lamotrigine, oxcarbazepine, tiagabine, topiramate, vigabatrin and zonisamide (4-11). While many of the new AEDs show improved efficacies and side-effect profiles, patients with intractable epilepsy remain untreated. There is clearly a need for improved medications, therefore, and an enormous effort has been exerted towards this goal over the last several years. Anticonvulsants were last reviewed in this series in 1989 (2). The purpose of the present report is to highlight the most important recent developments in antiepileptic drug research, with an emphasis on the discovery of novel therapeutics and the elucidation of their mechanism of action.

Until recently, AEDs were identified through a combination of empirical compound screening and serendipitous discovery. Intensive research into the physiological and biochemical events which take place during epileptic seizures has provided insight into the molecular mechanisms by which these might be controlled (12,13). The present understanding of the mechanisms by which AEDs exert therapeutic benefits, and consequently the way in which this chapter is organized, may be summarized as follows: (a) by potentiating inhibitory mechanisms (predominantly the GABA system); (b) by inhibiting excitatory mechanisms (predominantly the glutamate system); and (c) by inhibiting excessive neuronal firing (modulation of membrane cation conductance *via* sodium, calcium or potassium channels). In addition, several drugs operate through other or unknown mechanisms. The mechanism of action of AEDs and their clinical significance in relation to animal seizure models has been reviewed recently (14).

Considerable progress has been made recently in understanding the underlying causes of epilepsy at a molecular level (15,16). Two point mutations in the cysteine proteinase inhibitor gene, cystatin B (CSTB), proved that this gene is responsible for progressive myoclonus epilepsy of the Unverricht-Lundborg type (17). The role of phosphoinositide-specific phospholipase C-β (PLC-β), a G protein-coupled enzyme, was studied with knockout mice (18). It was found that PLC-β1$^{-/-}$ mice developed epilepsy and that PLC-β1 is involved in signal transduction by coupling to muscarinic receptors. Neuronal apoptosis was observed in the rat dentate gyrus in two models of human limbic epilepsy (19).

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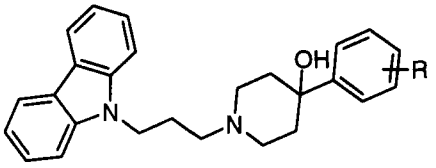
DRUGS THAT POTENTIATE INHIBITORY MECHANISMS

A number of newer AEDs enhance γ-aminobutyric acid (GABA) activity as a primary mode of action (20). The steps in the synaptic actions of GABA which are possible sites of drug intervention include stimulation of GABA release, inhibition of GABA reuptake, reduction of the breakdown of GABA, or modulation of post-synaptic GABA receptors (GABA-R). GABA-R are comprised of $GABA_A$-R, which are ionotropic and coupled to fast chloride channels, and the metabotropic $GABA_B$-R, which are linked *via* G proteins to potassium (K^+) and/or calcium (Ca^{2+}) channels and adenylate cyclase (20,21).

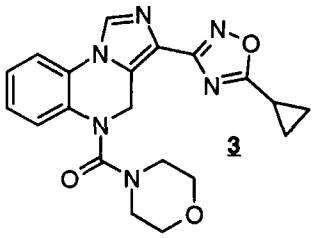
There have been extensive efforts to further elucidate the role of GABA-R in epilepsy (20). Thus, knockout mice, in which the $GABA_A$-R β3 subunit was inactivated by gene targeting, displayed signs of neurological impairment including epileptic seizures (22,23). The expression of $GABA_A$-R mRNA was measured in hippocampal slices prepared from rats with pilocarpine-induced seizures (24). Reduced levels of mRNA expression for the α2 and α5 subunits were evident in the hippocampus of epileptic animals, but no decrease in mRNA encoding α1, α2, or γ2 subunits was observed. Mice bred to be deficient in the 65-kDa isoform of glutamic acid decarboxylase (GAD65), which synthesizes GABA *in vivo*, developed seizures precipitated by fear or mild stress, indicating that $GAD65^{-/-}$ mice represent a useful model of epilepsy (25).

GABA Uptake Inhibitors - Tiagabine is a lipophilic analogue of nipecotic acid which selectively inhibits GABA uptake [GABA transporter I (GAT-1) antagonist] (9). It exhibits anticonvulsant effects in both amygdala- and hippocampal-kindled seizures in rats, antagonizes pentylenetetrazole (PTZ)- and 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM)-induced tonic convulsions, and sound-induced seizures in DBA/2 mice (9,26-28). Two tiagabine analogues, NNC 05-2045 (**1**) and NNC 05-2090 (**2**), blocked audiogenic seizures in DBA/2 mice and genetically epilepsy-prone (GEP) rats (**1** only), and were anticonvulsant in the maximal electroshock seizures (MES) test and in amygdala kindled rats (29).



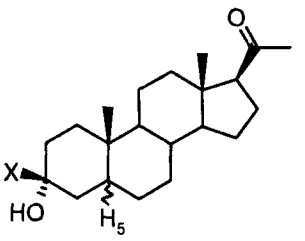
1 R = 4-OMe
2 R = 2-OMe

Benzodiazepine Receptor Modulators - Benzodiazepines and other ligands that interact at the $GABA_A$/benzodiazepine receptor complex (BzR) span a continuum of activity ranging from full agonists through antagonists to inverse agonists. A series of imidazoquinoxaline amide, carbamate and urea derivatives that exhibit high affinity for the $GABA_A$/benzodiazepine complex were investigated (30-32). Thus, screening against [^{3}H]-flunitrazepam led to identification of **3** with an IC_{50} of 0.81 nM, which was effective in antagonizing PTZ-induced seizures following intraperitoneal (ip) administration in mice with an ED_{50} value of 0.84 mg/kg.



3

Neuroactive steroids – Subcutaneous (sc) administration of 3β substituted steroid ganaxolone (**4**) was shown to protect against PTZ and cocaine-induced convulsions in mice (ED_{50} values of 3.45 and 7.78 mg/kg respectively). Ganaxolone (sc) also potentiated the anticonvulsant effect of diazepam (0.1 mg/kg) against PTZ with an ED_{50} value of 0.97 mg/kg (33). 3β-Phenylethynyl derivatives, such as **5**, were active in the rodent PTZ and MES tests following ip administration with ED_{50} values for **5** of 2.8 and 9.2 mg/kg, respectively (34).

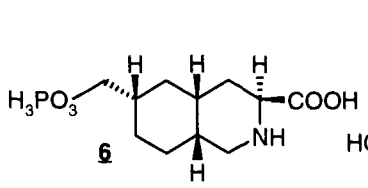


4 X = Me, H_5 = α
5 X = C≡C-4-(C(O)Me)-Ph, H_5 = β

DRUGS THAT INHIBIT EXCITATORY MECHANISMS

Compounds which modulate glutamic acid receptors are frequently first evaluated for anticonvulsant activity. Glutamate receptors fall into two main classes which encompass ion channels (NMDA, kainic acid/AMPA receptors) and metabotropic receptors (mGluRs) (35).

Ion-channel glutamate receptors (NMDA) - The status of the discovery of NMDA antagonists has been reviewed recently (36). Despite the fact that NMDA channel blockers have generally failed in the clinic due to unacceptable side effects, considerable research continues in this area, and many compounds have been evaluated in epilepsy models. It was reported that the competitive NMDA antagonists, LY235959 (**6**) and LY233053 (**7**) enhance the anticonvulsant activity of various drugs in the mouse MES model of epilepsy (37). Another non-competitive antagonist, the sterol hemisuccinate derivative **8**, was neuroprotective against NMDA-induced seizures in mice (38).



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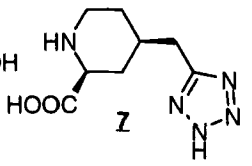

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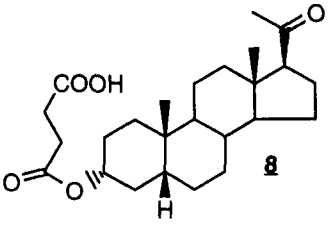
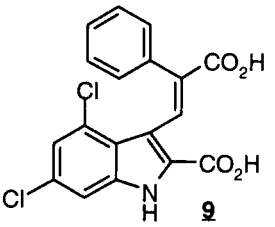


8

A number of allosteric modulators acting at the glycine binding site of the NMDA receptor complex continue to show antiepileptic potential. The indole **9** (MDL 105,519) showed anticonvulsant activity in a number of models (39); the closely related **10** (GV150526) similarly attenuated NMDA-induced seizures (40). The anticonvulsant activity of quinolinones, such as **11** (L-701,324) has been confirmed (41) and a large series of analogues were reported (42). Compound **12** (ACEA-1416), the 6-methyl-7-chloro analogue of **13** (ACEA-1021), and related benzazepines, such as **14**, were active in attenuating electroshock-induced seizures (43-45).

Ion-channel glutamate receptors (AMPA/Kainate) - The therapeutic potential of AMPA/Kainate receptor antagonists has been reviewed recently (46). Although compounds with anticonvulsant activity have been synthesized, to date none appear to have a therapeutic range to suggest clinical utility, due to adverse effects on motor performance (47). In an attempt to improve the physical properties of quinoxaline-



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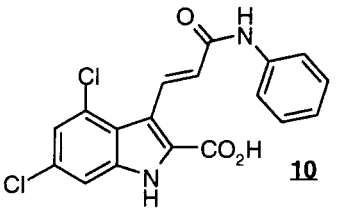

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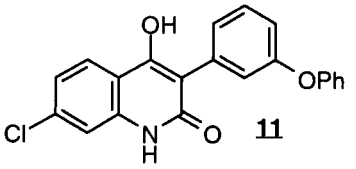

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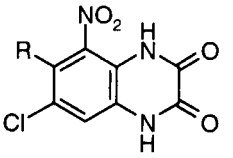
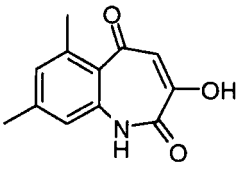
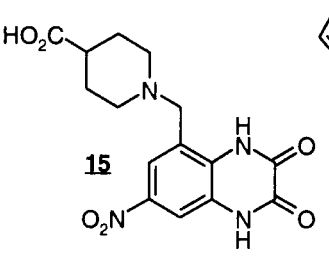


12 R = CH_3
13 R = Cl



14

diones, a series of 5-substituted derivatives led to the identification of 15 as a hydrophilic antagonist active in the MES model (48,49). The anticonvulsant activity of YM 90K (16) has been studied in some detail (50) and it was reported that introduction of an additional nitrogen atom led to elimination of affinity for the glycine site of the NMDA receptor; 17 was a potent AMPA antagonist with good activity in the DBA/2 mouse epilepsy model (51). *N*-Hydroxylation led to a very potent AMPA antagonist, 18, with over 100-fold selectivity in affinity compared to the NMDA glutamate and glycine binding sites (52).



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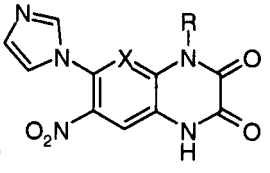
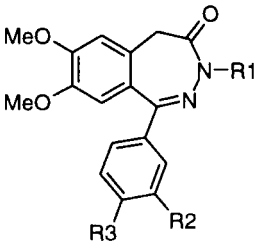
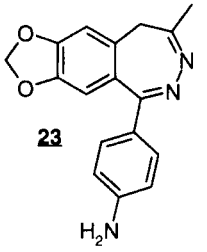


16 X = CH; R = H
17 X = N; R = H
18 X = CH; R = OH

Analogues 19-22 of the non-competitive benzodiazepine AMPA antagonist GYKI 52466 (23), were reported to be equally active in a number of epilepsy models with lower toxicity than the parent (53). The related dihydrophthalazines 24 were reported to have a similar profile, but with no activity at the central benzodiazepine binding site (54). The phosphonic acid 25 is an orally active, water soluble, selective



19 R1, R2, R3 = H
20 R1= CH_3; R2, R3 = H
21 R1, R3 = H; R2 = NH_2
22 R1, R2 = H; R3 = NH_2



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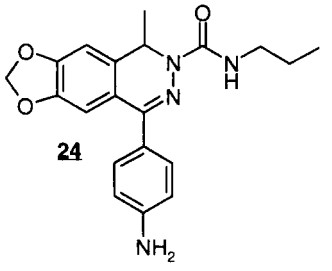

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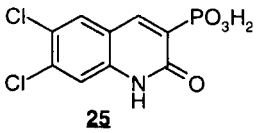

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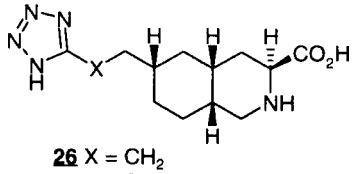
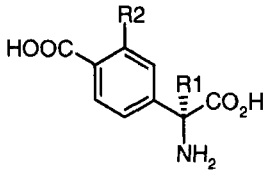


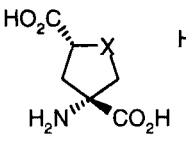
26 X = CH_2
27 X = S

AMPA/kainate antagonist with good activity in attenuating audiogenic seizures (55). Further SAR results of the decahydroisoquinoline derivative, LY293558 **26** and related compounds such as **27** were reported (56,57). Some of these antagonists were active in epilepsy models with reduced side-effects.

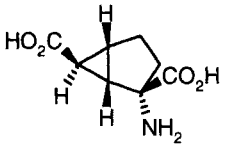
Metabotrophic glutamate receptors (mGluRs) - It has become apparent that modulators of the G protein-linked mGluRs have potential as antiepileptic agents. Activation of Group II/III or antagonism of Group I receptors (58) is expected to be reflected in activity in animal models of epilepsy (59). Thus, compound **28** [(*S*)-4C3HPG], which is both an agonist at Group II/III and antagonist at Group I, is active in the PTZ and DMCM epilepsy models (60). Phenylglycine derivatives, such as **29**, selectively attenuated Group III receptors and were active in the DBA/2 mouse model. Compound **30**, a non-selective agonist, is active in the DBA/2 mouse (60) and amygdala kindled rat models (61). Introduction of nitrogen in the cyclopentyl ring (62) furnished the selective Group II agonist **31**. Elaboration of this compound, based on elegant molecular modeling, led to the design and synthesis of bicyclic analogues, including **32** (LY354740), which is a potent, selective Group II agonist with anticonvulsant activity in the ACPD-induced limbic seizure model (63). Another conformationally-restricted analogue, **33** (DCG-IV) similarly activated Group II mGluRs and was active in the kainic acid seizure model (64).



28 R1 = H; R2 = OH
29 R1 = CH_3; R2 = H



30 X = CH_2
31 X = NH



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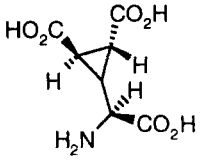

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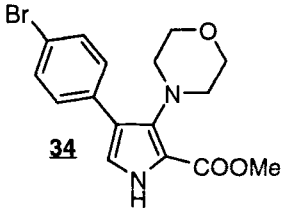
DRUGS THAT INHIBIT EXCESSIVE NEURONAL FIRING

Inhibition of neuronal cation conductance *via* block of voltage-gated sodium channels (VGSC) is a proven mechanism by which certain drugs act to control seizures (for a review of VGSC see Chapter 6 of this Volume). In addition, recent evidence points to a significant role for neuronal voltage-gated calcium channels (VGCC) as molecular targets for the treatment of epilepsy (65).

Inhibition of VGSC - The established first choice AEDs phenytoin, carbamazepine, and valproic acid, exert their effects primarily by antagonism of neuronal VGSC (13,66,67). Many of the newer drugs, including lamotrigine (68), felbamate (69), and topiramate (70) exhibit significant inhibitory activity at VSSC in addition to other modes of action. The pharmacology and pharmacokinetics of fosphenytoin, a phosphate ester prodrug of phenytoin developed as a replacement for injectable sodium phenytoin, have been reviewed recently (8,71). Oxcarbazepine is functionally a prodrug of carbamazepine with fewer side effects than the parent (4,11).

A series of sodium channel blocking 3-amino-pyrroles was synthesized and tested for anticonvulsant activity (72). Compound **34** was orally active in the MES test in rats (ED_{50} value of 2.5 mg/kg) with no neurotoxicity noted, even at high doses. Three vinca derivatives,



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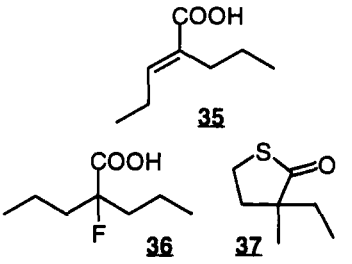
vinpocetine, vincamine and vincanol, were found to attenuate MES-induced convulsions in mice (ED_{50} values of 27, 15.4 and 14.6 mg/kg ip respectively) and block whole-cell Na^+ currents in rat cortical neurons with IC_{50} values of 44, 72 and 40 μM respectively (73). A metabolite of valproic acid (VPA), *trans*-2-ene valproic acid (**35**), was found to limit sodium-dependent action potential firing rates recorded in cultured mouse spinal cord and cortical neurons (74). Pharmacokinetics and metabolism studies were performed on an α-fluorinated analogue of VPA (**36**) which is anticonvulsant in the PTZ model with an ED_{50} of 1.7 mmol/kg ip (75).

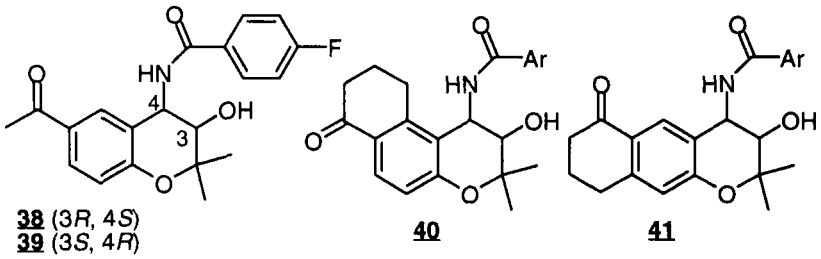


Inhibition of VGCC - Several AEDs, including VPA, ethosuximide, dimethadione and zonisamide are antagonists of T-type calcium channel currents (4,10,13). Furthermore, the anticonvulsant thiobutyrolactone α-EMTBL (**37**) reduced T-type currents in rat dorsal root ganglion neurons (76). Lamotrigine has recently been shown to inhibit N-type (65,77) and P/Q-type calcium channels (65), while ω-conotoxin MVIIC and ω-agatoxin IVA, which act predominantly at P/Q-type calcium channels, prevented clonic and tonic sound-induced seizures in DBA/2 mice (78). A mutation identified in the α_{1A} VGCC subunit gene of *tottering* and *leaner* mice was related to the seizure phenotype displayed by these strains (79). During the initial stages of hippocampal-kindling induced in rat, α_{1A}-, α_{1D}- and α_{1E}-VGCC subunit mRNA levels were significantly increased, in contrast to α_{1B}-subunit gene expression which decreased and α_{1C} in which no significant change was observed (80).

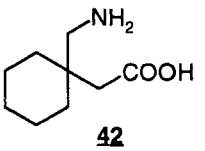
DRUGS WHICH ACT THROUGH OTHER OR UNKNOWN MECHANISMS

Despite the recent progress in understanding the biochemistry of epileptic seizures there continues to be therapeutic agents discovered for which there is, as yet, no clear mechanistic rationale. Research programs based on well defined points of intervention have in many instances resulted in identification of anticonvulsants with ambiguous or multiple modes of action. This section summarises the advances made in areas of research not already covered in the previous sections.

Compound **38** (SB-204269), which resulted from a program focused on potassium channel modulators, is an orally active anticonvulsant in the mouse MES test at 0.1-30 mg/kg (81). A novel, stereoselective binding site was identified in rat brain membranes using [^{3}H]-SB-204269 with 1000-fold difference between the affinities of SB-204269 (82) and the antipode **39**. In order to elucidate the conformational preference of the acyl moiety, racemic fused-ring ketones were prepared (83). It was found that **40** retained potency in a binding assay against [^{3}H]-SB-204269 while **41** was essentially inactive, suggesting that **40** mimics the low



energy conformation at the binding site.



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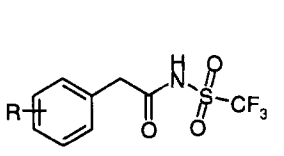
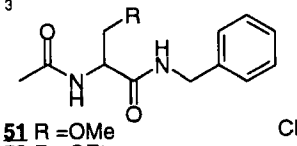
Originally designed as a GABA mimetic, gabapentin (**42**) does not interact with any known sites on GABA-R, nor does it block GABA uptake or inhibit GABA transaminase (84). Radioligand binding studies with [^{3}H]-gabapentin have established that gabapentin binds to the $\alpha_2\delta$ VGCC subunit; however, the significance of this finding in relation to the mechanism of action of the drug is unclear. A series of conformationally biased analogues of gabapentin was prepared in order to determine a conformational preference for the drug in its binding interactions with the $\alpha_2\delta$ subunit (85).

The pharmacological and clinical profiles of levetiracetam, an anticonvulsant analogue of the nootropic agent piracetam were recently reviewed (86), as were the antiepileptic effects of adenosine, ATP and adenosinergic ion channel modulators (87). The anticonvulsant losigamone (AO-33) is a β-methoxy butenolide derivative in clinical trials for the treatment of epilepsy. A study of its mechanism of action focused on the effects of the drug on NMDA- and AMPA-induced depolarizations in a cortical wedge preparation of the DBA/2 mouse and on amino acid release from BALB/c mouse cortical slices (88).

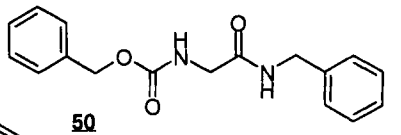
A series of *N*-(phenylacetyl)trifluoromethanesulfonamides (**43**-**49**) was prepared in order to evaluate the effect of aryl substituents on anticonvulsant activity (89). All seven compounds exhibited significant activity against MES- (ED_{50} value of 6.2 mg/kg ip for **43**) and PTZ-induced seizures; *in vitro* binding studies revealed that **43** did not displace [^{3}H]-GABA or [^{3}H]-flunitrazepam. Ester and amide derivatives of *N*-(benzyloxycarbonyl)glycine as well as esters of *N*-(3-phenylpropanoyl)glycine were prepared and evaluated in the MES test and several chemically induced seizure models (90). Benzylamide **50** had an ED_{50} of 4.8 mg/kg ip in the MES test. Derivatives of *N*-benzyl-2-acetamidopropionamide, **51** and **52**, were highly potent in the MES test, exhibiting ED_{50} values of 8.3 and 17 mg/kg ip,respectively (91). A series of (aryloxy)aryl semicarbazones were synthesized and evaluated in MES, PTZ, and neurotoxicity (NT) screens (92). Quantitative structure-activity relationship analysis, and X-ray crystallography of five compounds indicated a number of physicochemical parameters, including interatomic distances and bond angles, which appeared to be important for activity in the MES test. Aroyl(aminoacyl)pyrroles such as **53** were anticonvulsant in the mouse and rat MES tests but not in the mouse metrazole test, a property in common with phenytoin and carbamazepine. Compound **53** exhibited ED_{50} values of 23.9 and 28.1 mg/kg ip in the mouse and rat MES tests, respectively (93).



43 R = H
44 R = 4-Cl
45 R = 4-Br
46 R = 4-OMe
47 R = 4-NO_2
48 R = 3-Cl
49 R = 3-OMe



51 R =OMe
52 R= OEt



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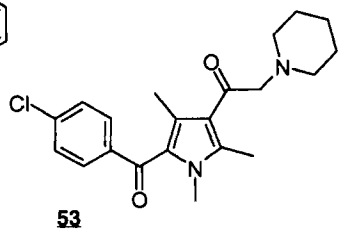

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Conclusion - An enormous quantity of research has been devoted to the study of epilepsy and antiepileptic agents over the last few years. This has led to significant advances in understanding the physiology of the disease state and consequently new and improved mechanisms of intervention. The older AEDs with broad activity profiles and adverse reactions in patients have given way to new AEDs with better defined mechanisms of action and fewer side effects. Further advances in molecular biology and physiology of epilepsies have provided even more insight, and the present generation of preclinical anticonvulsants will likely lead to highly selective, well tolerated AEDs of the future.

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SECTION II. CARDIOVASCULAR AND PULMONARY DISEASES

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Chapter 8. Recent Advances in Neurokinin Receptor Antagonists

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Introduction - The structurally related neuropeptides, substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), belong to a family of molecules termed tachykinins (TKs). Widely distributed in the central and peripheral nervous systems, TKs influence function of many tissues. SP and NKA have been linked to several chronic diseases: asthma, inflammatory bowel disorders, rheumatoid arthritis pain and psychiatric disorders (1 - 4). Less is known of the actions of NKB; however, cough behavioral and cardiovascular responses have been indicated (5, 6). The role of these neuropeptides in pathophysiology has been advanced by the recent development of antagonists selective for each of the neurokinin receptors (termed NK1, NK2, and NK3 for their preference for SP, NKA, and NKB, respectively).

Tachykinin research has been reviewed extensively (for recent examples, see (7-14)). Following a brief discussion of recent results on TKs in inflammation (an underlying feature of many chronic diseases) this chapter reviews recent advances in the development of the neurokinin antagonists. Whenever possible, the literature cited here will reflect work performed since the publication of a similar review (15).

TACHYKININS AND INFLAMMATION

Expression of tachykinins and their respective receptors is altered by a number of inflammatory conditions including arthritis and asthma. SP and NKA are encoded from a single gene (PPT-A) which gives rise to multiple mRNAs, i.e., α, β, and γ, through RNA splicing (16). The amounts of these precursors are regulated normally in a tissue-specific manner which determines local levels of the neuropeptides (for review, see (1)). The NKB gene (PPT-B) shows structural similarity to PPT-A; however, the PPT-A and -B mRNAs differ in the major sites of their expression (17). Enhancement of afferent input (for example, in chronic polyarthritis) increases the levels of SP, its transcript and NK1 receptors in neurons of the dorsal horn (the first synaptic processing station for pain) and in those neurons proximal to the site of input (18, 19). Spread of inflammation contralateral to the initial arthritic joint also gives rise to dorsal horn expression of PPT-A (20). In human asthma, mRNA expression of NK1 and NK2 receptors increases(21, 22). Exposure of animals to allergen enhances levels of PPT-A in the nodose ganglion (the major sensory supply to the airways) concomitant with a 3-4 fold rise in SP and NKA in the lung (23). Mice over-expressing nerve growth factor (selective for lung) demonstrate tachykinin positive hyperinnervation and increased SP content in the lung with associated airway hyperreactivity , characteristic of asthma (24). Thus, plasticity of TK (and their respective receptors) expression influences organ function in animal models.

SP may exacerbate inflammatory states by promoting the migration of leukocytes (13). Local administration of SP to normal human skin and other tissues, induces rapid and prolonged influx of neutrophils and eosinophils into the dermis (25, 26). Adhesion of granulocytes is prevented by NK1 receptor antagonism or antibodies to ICAM-1 which is upregulated on endothelium by SP (27- 29). SP therefore stimulates formation of gaps between endothelial cells, which is permissive to migration of inflammatory cells, and it propagates chemoattraction of these cell types (30).

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Tachykinergic fibers extend to primary and secondary lymphoid tissues which contain NK1 receptors and thereby link TKs to immune aspects of inflammation (31-33). Immune cells; T-lymphocytes (34), B cells, and hematopoietic progenitors (for review, see (35)), express NK1 receptor mRNA. Lymphocyte proliferation and function, i.e., cytokine production, is stimulated by SP; IL-2 in T-cells, and IL-1, IL-3, IL-6 and GM-CSF in bone marrow mononuclear cells (36 - 38). Activation of NF-κB (a transcription factor involved in cytokine expression) is triggered with nM concentrations of SP and is blocked by a NK1 receptor antagonist (39). Alveolar macrophages express PPT mRNA and respond to both SP and NKA by producing superoxide anion via the respiratory burst (40, 41). Macrophages activated in vivo by SP also secrete TNFα, suggesting a potential autocrine or paracrine role for SP in sites of inflammation (42). Finally, molecular disruption of the NK1 receptor protects the lung from immune complex injury (43).

Recent studies using "knock-out" mice (lacking either TK production or the NK1 receptor) have provided new information on the role of TKs in pain transmission and, to date, one other inflammatory condition (enteritis (44)). Previous work suggested that tolerance to painful stimuli was achieved from depletion of neurokinins from C-fiber afferents and induction of hyperalgesia required activation of both NK1 and NK2 receptors (45 - 47). Maintenance of hyperalgesia involves only those neurons in dorsal horn expressing the SP receptor (48). Mutant mice lacking the PPT-A gene (no detectable levels of SP and NKA) are hypoalgesic within a specific "window" of pain intensities; i.e., moderate to intense pain, independent of pain modality (49); for alternative viewpoint, see (3). Interestingly, stimuli sufficient to provoke neurogenic inflammation in wild type mice was essentially without effect in TK null mice (49). Moreover, animals without NK1 receptor demonstrate blunted responses to intense noxious stimuli and were free of the characteristic amplification (so-called "wind up") to painful stimuli (50). These observations are timely given recent discovery of potent NK, especially NK1, receptor antagonists.

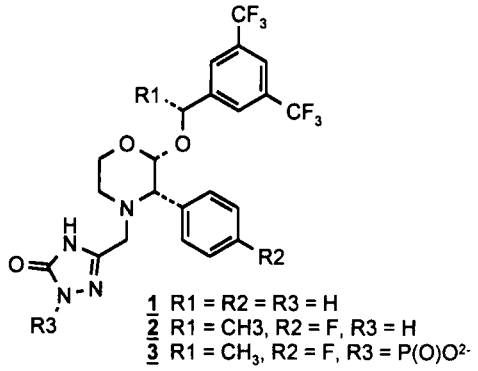
NK1 ANTAGONISTS

New Entities - Efforts to improve metabolic stability and potency of the previously reported NK1 antagonist L-742,694 (**1**) (51), led to synthesis of L-754,030 (**2**) (52). The phosphonate amide prodrug L-758,298 (**3**) (53) and another class of prodrug where R3 = CO2CH2CH2NH2·HCl (54) have improved water solubility. The role of (S) α methyl benzyl moiety is more fully described in a related piperidine series (55). A recently reported alicyclic derivative of L-742,694, (**4**), is selective for the NK1 receptor and displays sub-nanomolar affinity (56).

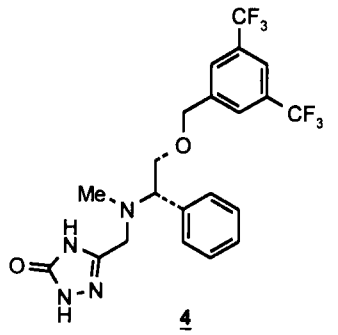
Compound **5** was the most active of a series of benzimidazolones in the hot plate test in mice (ED_{50} 0.3 mg/kg po). Compound **6** was active in a mouse SP-induced bronchial hypersensitivity model (ID_{50} 3mg/kg po) and active iv in a guinea pig SP-induced bronchoconstriction model (57).

Using a unique synthetic approach, a template for azanorbornane derivatives that display potent SP receptor affinity has been prepared (58). Of the compounds reported, **7c** is the most potent with a Ki of 0.16 nM against human NK1 receptor. SAR studies and conformational analysis were used to optimize NK1 activity in a previously reported isoquinoline and pyrido[3,4-b]pyridine series (59); the tetrahydropyridines **8a**, **8b** and **8c** exhibit excellent in vitro and in vivo inhibitory activity (ED_{50} 0.20-0.27 mg/kg, po - capsaicin-induced plasma extravasation in guinea pig trachea) (60). In the same animal model, a related 1,7-naphthyridine has oral activity (ED_{50} 0.068 mg/kg) (61). The most active of a series of 1,4-benzodiazepin-2-one derived NK1 antagonists is **9** with a Ki of 10 nM (62). SAR studies on the previously reported CGP49823 (**10**) (63) yielded CGP73400

(**11**) with an improved oral activity (ED_{50} in a NK1 bronchospasm model 4.4 and 0.19 mg/kg po, respectively) (64). MEN 10930 (**14**, X=1,1-cyclohexyl) is a potent, specific and competitive inhibitor of the human NK1 receptor (Ki=1.0 nM) but shows essentially no affinity for NK1 sites present in rat urinary bladder membranes (65). Administration (iv) of a related pseudopeptide, MEN 11149 (**14**, X= (1R, 2S)-cyclohexyl) prevented plasma protein extravasation induced by [Sar^9,Met(02)^{11}]-substance P in guinea pig bronchi and nasal mucosa (66, 67). The duration of inhibition (sc) in nasal mucosa was greater than 6 hours.



1 R1 = R2 = R3 = H
2 R1 = CH3, R2 = F, R3 = H
3 R1 = CH_3, R2 = F, R3 = $P(O)O^{2-}$



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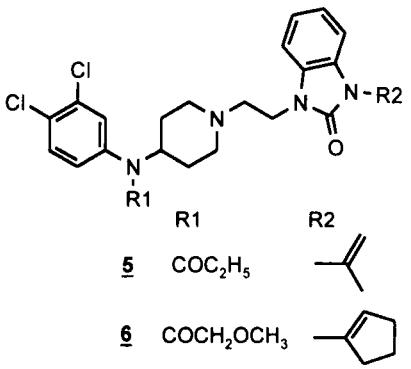
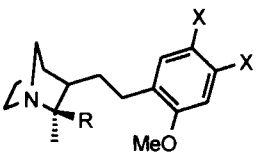
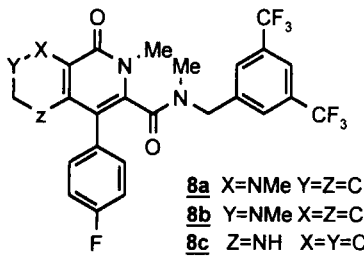


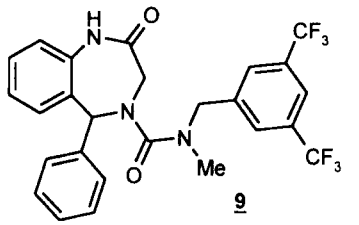
	R1	R2
5	COC_2H_5	
6	$COCH_2OCH_3$	



			Ki (nM)
7a	R = Ph	X = H	79
7b	R = $CHPh_2$	X = H	0.3
7c	R = $CHPh_2$	X= Me	0.16



8a X=NMe Y=Z=CH_2
8b Y=NMe X=Z=CH_2
8c Z=NH X=Y=CH_2



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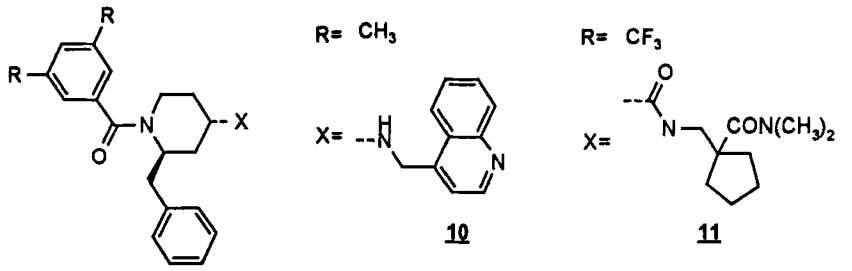
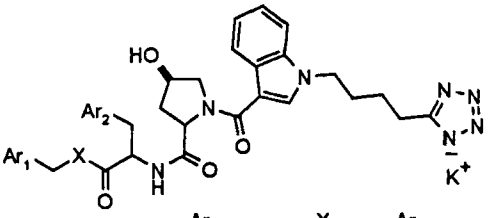
Studies Involving NK1 Antagonists - Molecular modeling studies that led to the attachment of the water-solubilizing butyl tetrazole group of S18523 (**12**) and S19752 (**13**) indicated that the tetrazole group lies outside the membrane-spanning domain of the NK1 receptor and does not significantly interfere with antagonist binding (68). Spectroscopic studies on a series of NK1 compounds of general formula **14** revealed that the extent of formation of a charge transfer complex between the indolyl and naphthyl rings correlates with hNK1 binding potency (69). Both CP-99,994 (**15**) and CP-96,345 (**16**) appear to bind within the NK1 receptor transmembrane domain bundle; however, the contribution of individual amino acid residues to the binding of each compound differs. In particular, **15** has interactions with Leu-203 and Ile-204 not present in the piperidine **16** (70).

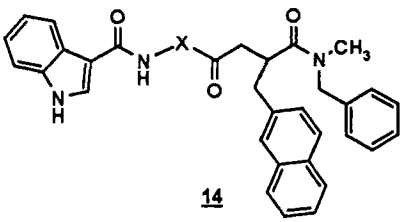


10 11

SR140333 (18) was active (icv) in a rat cerebral ischemia model, suggesting that NK1 antagonists may find utility as treatment of cerebral ischemia (71). Compound 18 was employed to show a preferential involvement of NK1 versus NK2 receptors in nociceptive transmission following trigeminal ganglion stimulation (72). In a ferret cisplatin-induced emesis model PD 154075 (19), which has good oral bioavailability and brain penetration, was effective against both the acute and delayed phases of emesis while ondansetron was potently effective only against the acute phase (73). Of interest, low bioavailability of Cam-2445 (20), which is highly lipophillic and poorly soluble, was due to precipitation within the GI tract (74).



	Ar_1	X	Ar_2
12	C_6H_4	NH	2-naphthyl
13	3,5-di$CF_3C_6H_3$	O	3-indolyl



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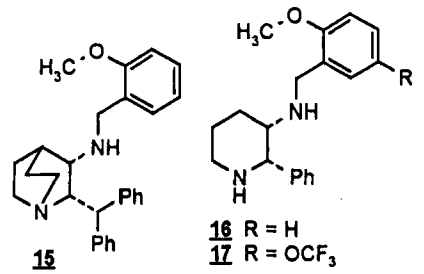
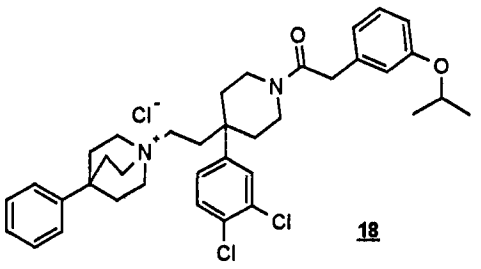
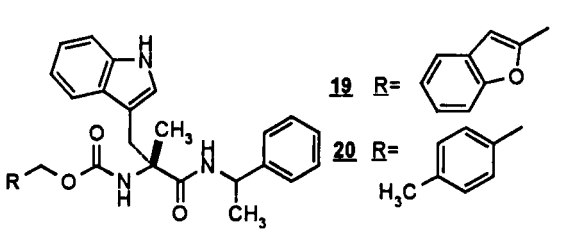


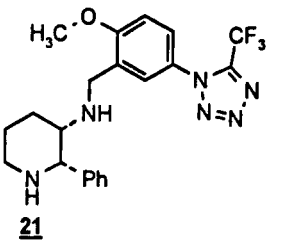
15 16 R = H
17 R = OCF_3



18

Clinical Studies Involving NK1 Antagonists - MK-869 (structure not revealed), has reportedly shown "excellent antidepressant and anxiolytic activity as monotherapy in a Phase II trial for depression" (75). For women after undergoing lower abdominal surgery CP122,721 (17) (200 mg po, 60-90 min prior to surgery) was equivalent to ondansetron (4 mg iv prior to end of the procedure) for control of nausea (76). However, those receiving 17 had a significantly lower number of emetic episodes. The combination of the two therapies provided the longest duration free of nausea and vomiting. GR205171 (21) (25 mg iv) was more effective than placebo in controlling post-operative nausea and vomiting in women following major gynecological surgery (77). There was, however, no relief of post-operative pain. Consistent with these findings, L-754,030 (2) did not prevent post-operative dental pain (78). In these trials, adverse effects have not been reported.



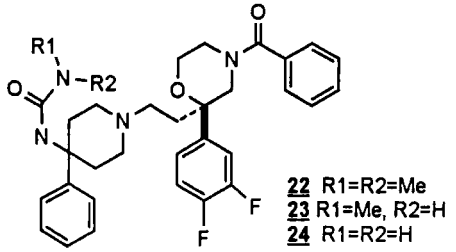


21

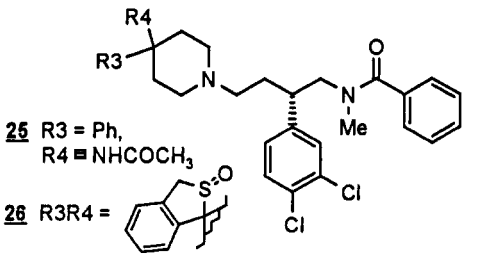
NK2 ANTAGONISTS

New Entities - The NK2 antagonist SR 144190 (**22**) blocked [βAla^8]neurokinin A-(4-10) induced bronchoconstriction in anaesthetized guinea pigs more potently than the prototype nonpeptide NK2 antagonist SR 48968 (**25**) after intravenous, intraduodenal or oral administration (79). The potencies of metabolites of **22**, SR 144782 (**23**) and SR 144743 (**24**), were similar when given iv or ip but were significantly less active following intraduodenal administration. Compound **22**, like **25**, antagonized citric acid-induced cough and airway hyperresponsiveness to acetylcholine. Additionally, **22** attenuated [βAla8]neurokinin A-(4-10)-induced urinary bladder contraction in rats, prevented castor oil-induced diarrhea in rats and blocked the turning behavior induced by intrastriatal injections of [Nle10]neurokinin A-(4-10) in mice (80).

YM-38336 (**26**) was equipotent to **25** in a NK2 binding assay but was 3-fold more potent in reversing [βAla8]NKA (4-10)-induced bronchospasm in guinea pigs (81). ZD7944 (**27**), a potent (Ki =1.1 nM) selective NK2 antagonist with high oral bioavailability antagonized aerosolized [βAla8]NKA (4-10)-induced dyspnea in conscious guinea pigs (>50% protection at 0.6 μmole/kg, po) (82). A NK2 lead **28** (isomer A, IC_{50} = 11 nM) was generated from a combinatorial library approach. Further biological evaluation of this series has yet to be published (83).



22 R1=R2=Me
23 R1=Me, R2=H
24 R1=R2=H



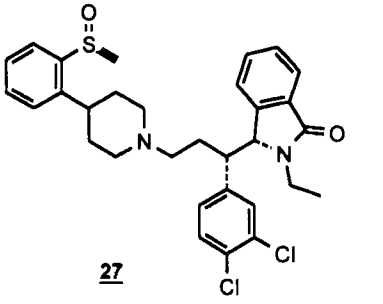
25 R3 = Ph, R4 = NHCOCH$_3$

26 R3R4 =

The introduction of a sugar moiety into the lipophilic MEN 10627 (**29**) to yield Nepadutant (MEN 11420, **30**) did not influence binding activity but markedly improved in vivo potency and duration of action (84). Compound **29** was shown to be a potent and selective antagonist of NK2 receptor mediated bronchoconstriction in guinea pig airways with a long duration of action (85).

Studies Involving NK2 Antagonists - From studies using compounds **22**, **23** and **24**, described above, the antitussive actions of NK2 receptor antagonists appear to be exerted via their effect on the peripheral nervous system (86). Alternatively, the site of action of **25** in the cat and guinea pig (capsaicin-induced cough) was shown to be centrally mediated (87). NKA induced hyperalgesia has been inhibited by **25** (88). Capsaicin induced urinary bladder motor responses was prevented by **30** (100 nmol/kg, i.v.) (89). Compound **30** blocked dose dependently contractile activity of human and guinea pig colon elicited by either NKA or the more NK2 selective agonist, [βAla8]NKA(4-

10) (90). It should be noted that under certain experimental conditions protection against gastric insults may be afforded by activation of NK2 receptors (91, 92).



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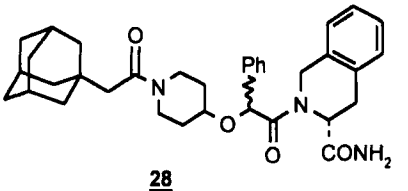

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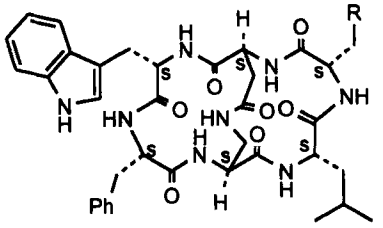
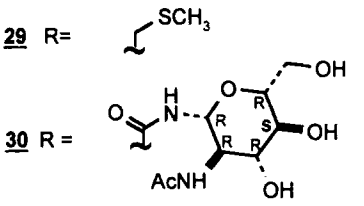


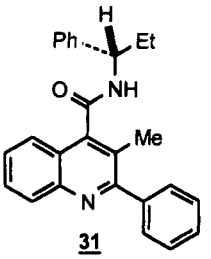


29 R=

30 R =

NK3 ANTAGONISTS

New Entities - SB 222200 (**31**) is a selective NK3 antagonist (Ki =4.4 nM) which blocked senktide-induced contraction of isolated rabbit sphincter muscle. Oral administration decreased s.c. senktide-induced head shakes and tail whips in mice (ED_{50} = 5.6 mg/kg). Compound **31** effectively crosses the blood brain barrier with brain concentrations 190% of plasma concentrations (93).



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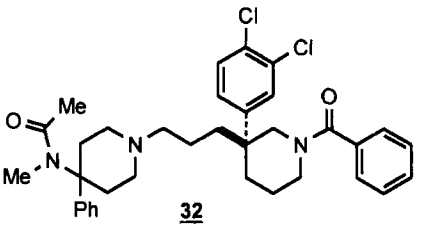


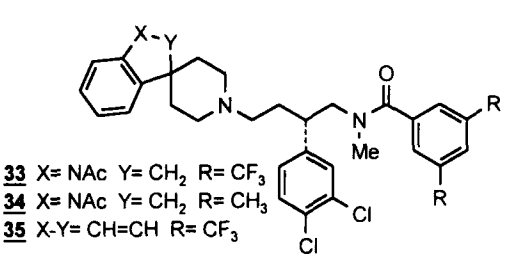
32

Studies Involving NK3 Antagonists - Studies using selective NK3 antagonists have been limited. A study with the NK3 antagonist SR 142801 (**32**) suggests that NK3 receptor stimulation might be involved in cough but not in bronchoconstriction induced by citric acid in guinea pig (94).

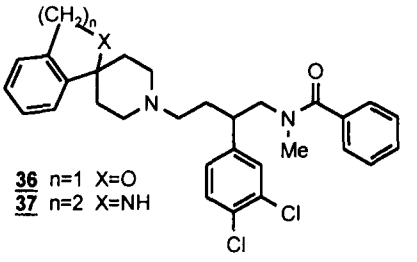
MIXED ANTAGONISTS

The spiro compounds **33** , **34** and L-743,896 (**35**), exhibit potent balanced NK1 and NK2 binding affinity and are orally active (95, 96). The spiro derivatives YM-35375 (**36**) and **37** are cited as lead compounds in a NK1-NK2 dual receptor antagonist program (81, 97). The SAR generated from a 20,000 member combinatorial library was used to design potent selective NK1-NK2 dual receptor antagonists (98). Seven NK receptor inhibitors, represented by SCH 60059 (**38**) and SCH 60065 (**39**), and two uncharacterized compounds, were isolated from fermentation broth. All of these compounds showed dual inhibition in NK1 and NK2 receptor binding assays (IC_{50} 2.5-11 μM for NK1 and 6.8-16 μM for NK2) (99). The synthesis, SAR and characterization of the previously reported

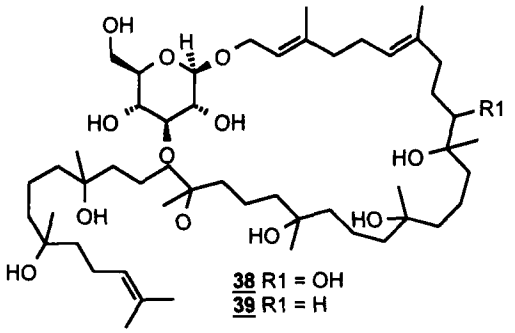
NK1-NK2 dual antagonist, MDL 105,212 (**40**), has been recently described (100). Compound **40**, given iv or po, inhibits capsaicin-induced bronchoconstriction in guinea pigs (101).



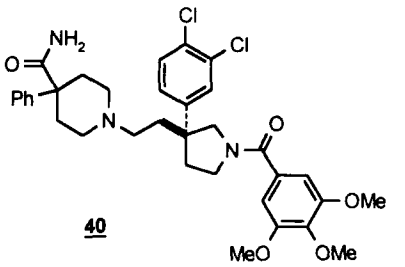
33 X= NAc Y= CH_2 R= CF_3
34 X= NAc Y= CH_2 R= CH_3
35 X-Y= CH=CH R= CF_3



36 n=1 X=O
37 n=2 X=NH

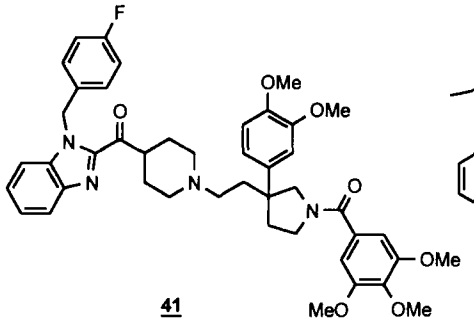


38 R1 = OH
39 R1 = H



40

Compounds **41** and **42** have been identified as dual histamine (H1) and NK2 receptor antagonists (102, 103).



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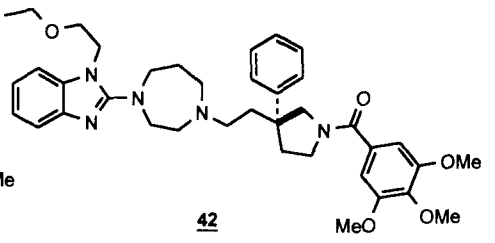

42

Conclusion - Pre-clinical data suggest a role for TKs in chronic inflammatory diseases. However, the outcomes of pilot pain trials using NK1 antagonists have been disappointing. To date, clinical evidence indicates that these agents will find application in conditions that lack an overt inflammatory component such as emesis and/or depression/anxiety. Nonetheless, there remains an enthusiasm for the anticipated clinical benefits afforded by NK antagonists in chronic inflammatory diseases such as asthma.

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Chapter 9. Thrombosis and Coagulation

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Introduction - A great deal of progress has been made in the past ten years towards developing agents for controlling thrombotic events. Treatment approaches include control of platelet aggregation, lysis of clots, and inhibition of thrombin generation and activity. This article will focus on advances towards discovering potent and specific thrombin and factor Xa inhibitors (1-5). Currently, heparin and warfarin are used for the treatment and prevention of thrombolytic events. Heparin is an intravenously administered compound which acts by inactivating various coagulation enzymes such as antithrombin III. Warfarin interferes with the synthesis of several enzyme precursors responsible for blood coagulation and generally takes several days for its effect to take place. Although both compounds are considered first line therapy for thrombolytic disorders, each has its limitations and there still exists a great need for a better anticoagulant. Hirudin and Hirulog are two direct acting thrombin inhibitors which have reached the clinical stage of development. Both compounds are large molecules and require intravenous or subcutaneous administration. Each compound has displayed good efficacy in small clinical trials but have limited utility due to the dosage requirements and safety considerations. For this reason a number of efforts have focused on finding small molecule inhibitors of the serine proteases thrombin and factor Xa. Thrombin cleaves a variety of substrates such as fibrinogen, factors V and VIII, factor XIII as well as the platelet thrombin receptor. Factor Xa is responsible for cleaving prothrombin to generate thrombin and control of this enzyme would have a direct effect on thrombin activity. Although several orally bioavailable small molecule inhibitors of thrombin have been developed, only a few of these have reached clinical trials and little is known about their efficacy. This article will focus on the progress made in discovering novel thrombin and factor Xa inhibitors within the past year.

LOW MOLECULAR WEIGHT-DIRECT ACTING THROMBIN INHIBITORS

Reversible Transition-State Inhibitors - The first synthetic serine protease inhibitors to be extensively explored were tripeptides such as **1** (6). These compounds are referred to as reversible transition-state inhibitors (RTSI) because they contain a C-terminal group which binds in a reversible fashion to the serine hydroxyl group at the enzyme active-site and mimics the transition-state achieved when the enzyme cleaves the substrate. Compound **2** was reported last year to be an example of an RTSI which contains a cyclic-lactam in place of the Phe-Pro dipeptide group normally found in inhibitors. A new series of inhibitors has recently been described in which the cyclic lactam has been replaced by a heterocycle (7). The compounds **3**-**5** are potent inhibitors of thrombin (IC_{50} = 0.47-2.32 nM) and show good selectivity (40-300 fold) towards the closely related enzyme trypsin. Modeling of these compounds into the thrombin active site suggest that the pyridinone group binds to the thrombin Gly-216 residue in a similar manner to the Phe-Pro group in standard inhibitors. The arginine-aldehyde group interacts with the serine hydroxyl and specificity pocket while the benzyl-sulfonamide group fills the hydrophobic S3 thrombin site. Compound **3** is also reported to be 31% orally bioavailable in dogs and to be efficacious in a rat thrombosis model.

Most thrombin inhibitors described to date also contain a basic moiety in the P1 sidechain which interacts with an aspartic acid residue (Asp189) found in the

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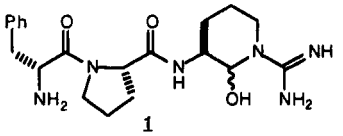
specificity pocket of thrombin. Boronic acid **6** has been reported to be a good thrombin inhibitor (IC_{50} = 6.3 nM) despite having a neutral P1 sidechain (8). Addition of a cyano group to the meta-position of the aromatic group further increases potency (**7**, IC_{50} = 0.48 nM) and it is also ten-fold selective over trypsin. An X-ray crystal structure of compound **7** bound to thrombin shows that the cyano-benzyl group does not fill the specificity pocket as anticipated but appears to swing away from the aspartic acid group. The cyano group forms a hydrogen bond with Gly219, which is a backbone residue of thrombin.



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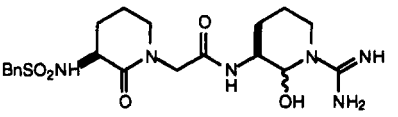
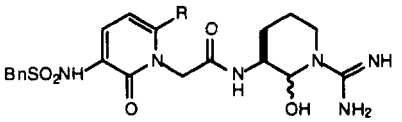
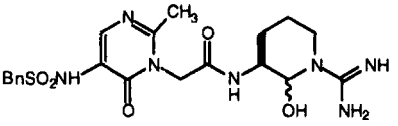


2 R = H IC_{50} = 6.2 nM

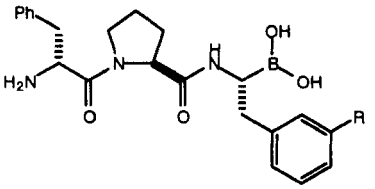


3 R = H IC_{50} = 0.505 nM

4 R = CH3 IC_{50} = 0.467 nM



5 IC_{50} = 2.32 nM

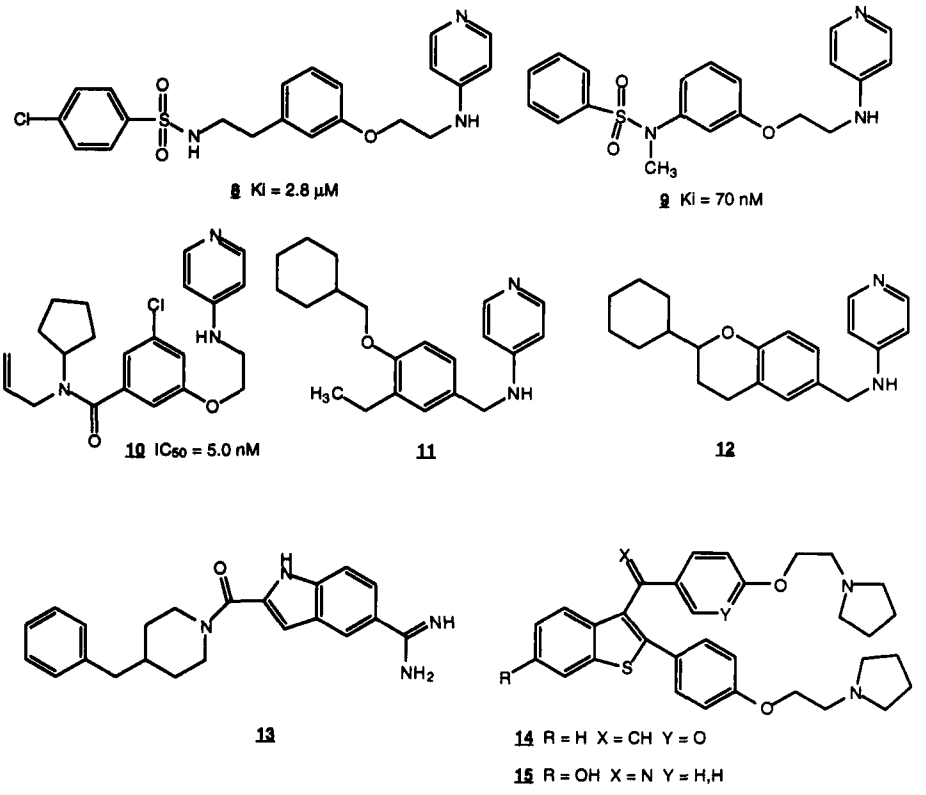


6 X = H Ki = 6.3 nM

7 X = CN Ki = 0.48 nM

Non-Peptide Derived Inhibitors - The past year has seen the disclosure of a variety of non-peptide derived inhibitors by several research groups. Most thrombin inhibitors are peptide derived and contain a highly basic residue in the P1 sidechain. These same features preclude most of these inhibitors from having good oral bioavailability in animal models. Compound **8** was discovered by screening compounds (against human thrombin) which contained groups with acidity constants of pKa < 10 (9). The 4-amino-pyridine group of **8** has a pKa of 9.2 and the compound is highly selective for thrombin. Exploration of the structure activity relationships of this novel series led to the discovery of compound **9**, which inhibits thrombin with an IC_{50} = 70 nM and is inactive against trypsin and plasmin. The reason for the high specificity of this compound might be explained after examining an X-ray structure of a closely related analog in the thrombin active site. The amino-pyridine group fits in the specificity pocket and has a favorable interaction with an adjacent alanine methyl group of the enzyme. In trypsin, the alanine is replaced by a serine and this would lead to an unfavorable hydrophilic and steric interaction. This 4-amino pyridine group has also been used in other inhibitor designs such as compounds **10** (IC_{50} = 5.0 nM), **11** and its constrained version **12** (10-12).

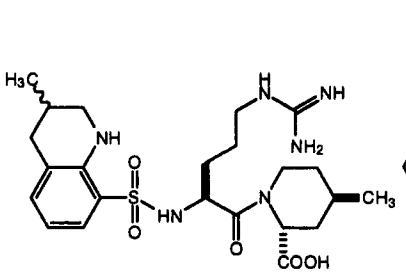
Examination of an X-ray structure of compound **13** bound to thrombin was recently reported (13). As expected, the benzamidine group forms a salt bridge with the aspartic acid group in the specificity pocket. The benzylpiperidine group spans the active-site cleft and interacts with the hydrophobic S3 site. There appears to be a



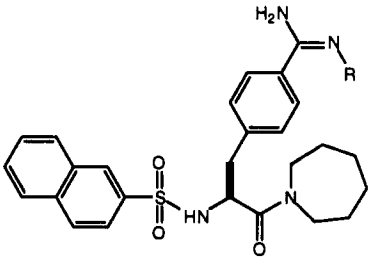
hydrogen bond between the catalytic serine hydroxyl group and the indole hydrogen. This interaction is seldom seen with other inhibitors.

Compound 14 was identified as a competitive thrombin inhibitor (Ki = 374 nM) through random screening of a sample collection (14). SAR studies showed that deletion of the ketone carbonyl group gave an eight-fold increase in potency. Addition of a 6-phenol led to a forty-fold increase in activity. Further modifications gave compound 15 which is a competitive (Ki = 9.9 nM) inhibitor, is selective against a variety of other serine proteases, and displays modest (9%) oral bioavailability in rats. An X-ray structure of 15 bound in the thrombin active site shows that the aryl portion of the thiophene sits deep within the specificity cavity and the phenol group interacts with Asp189. The side chain spans the S2-S3 site and the pyrrolidine group interacts with the hydrophilic S3 pocket. The C2 side chain has few interactions with the active site and predominantly resides in solvent.

Inhibitors Based Upon Argatroban and NAPAP - Argatroban (16) differs from the previously described inhibitors in that it is comprised of only two amino acids and that the basic amine sidechain interacts with the specificity pocket through a different approach (15). The compound is in phase III clinical trials in the U.S. and is already approved for use in Japan as an intravenous agent. Compound 17 was designed based upon the argatroban template and is a modestly active inhibitor of bovine thrombin (Ki = 52.4 nM) with poor selectivity for trypsin (16). It was hypothesized that selectivity over trypsin might be achieved by increasing the size of the group which fills the specificity pocket. Since thrombin has a larger pocket than trypsin, this should engender specificity over trypsin and this was indeed the case for the amidrazone 18 (Ki = 1.47 nM, 20 fold increase in specificity). Compound 19 is a

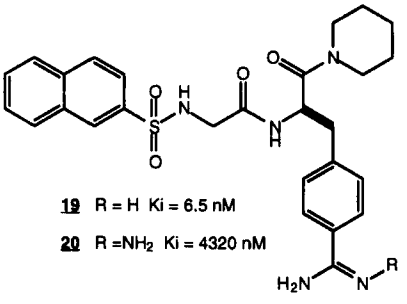


16 Argatroban Monohydrate



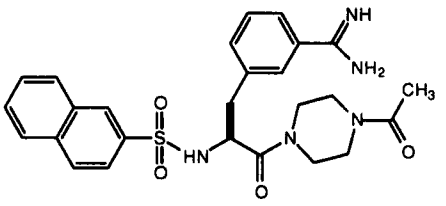
17 R = H Ki = 52.4 nM

18 R =NH_2 Ki = 1.47 nM

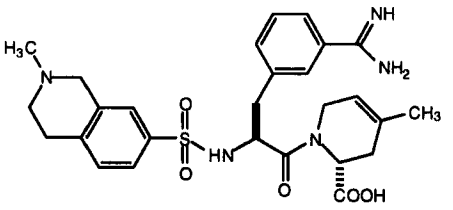


19 R = H Ki = 6.5 nM

20 R =NH_2 Ki = 4320 nM



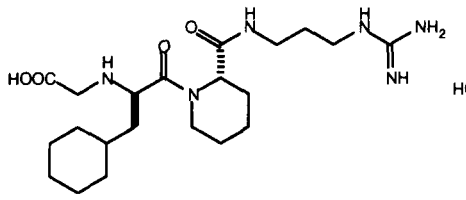
21 Ki = 23 nM



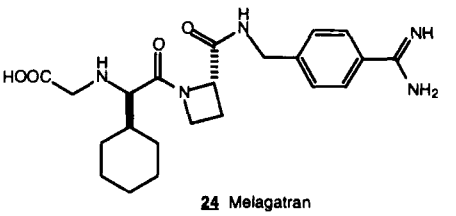
22 UK156,406 Ki = 0.46 nM

potent thrombin inhibitor (Ki = 6.5 nM) but unlike before, activity is lost when the extra amine group is added to the amidine moiety (**20**, Ki = 4320 nM). It is postulated that the benzamidine group in compound **18** enters the specificity pocket from a different angle and the addition of the extra amine causes an unfavorable interaction with the enzyme. It was recently reported that substitution of the N-terminal aza-cycloheptane group of compound **18** with an N-methyl-N-cyclopropyl group affords LB30057 which is potent (Ki = 0.38 nM) and has good oral bioavailability (58%) in dogs (16). Compound **21** was designed based upon the NAPAP series of compounds and is reported to be potent (Ki = 23 nM), selective (6-fold) and to possess oral bioavailability in rats, however, duration remains a problem (18). Compound **22** (UK156,406, Ki = 0.46 nM) is a similar structure and has recently entered clinical trials. The compound was reported to be well absorbed and tolerated in normal healthy volunteers after the administration of single doses in the range of 10-200 mg. However, duration may be an issue because the anticoagulant parameters returned to baseline within 8 hrs of the dose administration (19).

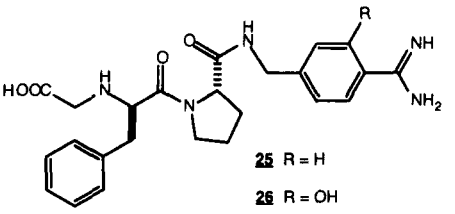
Tripeptide Derived Non-Covalent Inhibitors - Compounds of this type consist of three residues which span the S1-S3 active sites. They usually contain a basic amine group in the P1 position which binds to Asp189 in the specificity pocket and do not react covalently with the active site serine group. A new efficient preparation of inogatran (**23**) which has undergone clinical evaluation as an intravenous agent, has been recently reported (20, 21). Modification of the P1-P3 portions of inogatran has resulted in the orally bioavailable compound melagatran **24** (22). The related



23 Inogatran

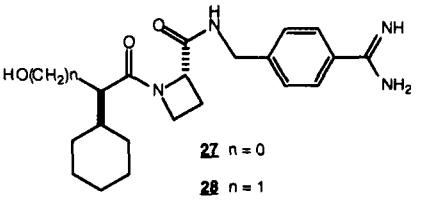


24 Melagatran



25 R = H

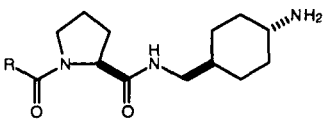
26 R = OH

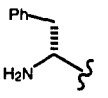


27 n = 0

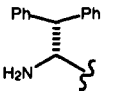
28 n = 1

derivative 25 is reported to be potent, selective and to have 22% oral bioavailability in rats (23). Selectivity versus trypsin can be improved by addition of a hydroxyl group adjacent to the amidine function to afford 26 (24). Compounds 27 and 28 represent examples of inhibitors which contain a hydroxyl group in the P3 residue in place of the acetic acid-amine normally found in inhibitors such as 23-26 (25).

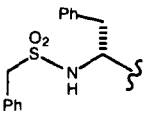




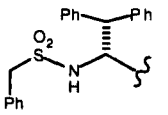
29 Ki = 5 nM



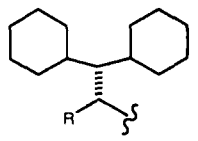
30 Ki = 0.1 nM



31 Ki = 0.4 nM

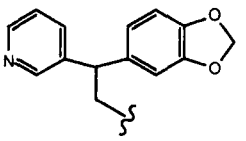


32 Ki = 0.0025 nM

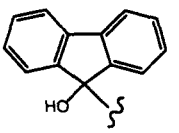


33 R = NH_2 Ki = 0.056 nM

34 R = H Ki = 14 nM



35 Ki = 0.28 nM

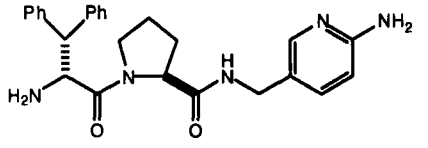


36 Ki = 1.5 nM

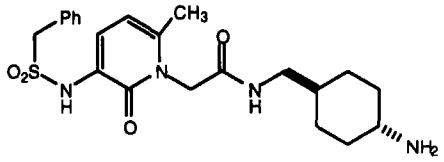
Compound 29 is a modest thrombin inhibitor with poor oral bioavailability in animals. Addition of a phenyl group to the P3 Phe sidechain led to a fifty-fold increase in activity (30, Ki = 0.1 nM) (26). A similar increase in activity was seen by appending a benzylsulfonamide group to the P3 Phe amine to give 31 (Ki = 0.4 nM). Compound 32 represents the combination of the two modifications and gave, as expected, a very potent compound (Ki = 0.0025 nM). An X-ray structure of 32 in the thrombin active site suggests that the benzylsulfonamide group occupies a novel lipophilic site formed by residues Asn217-Gly219 and the cyclohexyl group of the inhibitor. It was found that reduction of the phenyl groups of 30 gives a potent compound 33 (Ki = 0.056 nM) which is selective (2100-fold) and orally bioavailable in dogs (90%) (27). However this

compound did not show good activity in an in vivo model and it was speculated that high protein binding may compromise its effectiveness in the presence of serum components. Further modifications in the P3 group resulted in the potent inhibitors **34** (Ki = 14 nM), **35** (Ki = 0.28 nM), and **36** (Ki = 1.5 nM) (28, 29). Compound **36** was discovered through a rapid multiple analog synthesis using a solid support approach. The compound has good oral bioavailability in animals (74% dogs; 39% rhesus monkeys) and occupies the thrombin active site in the usual fashion.

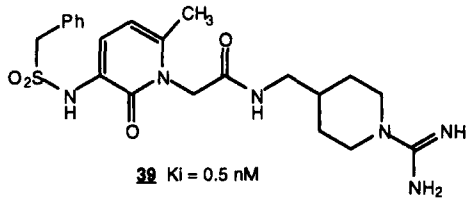
Inhibitors which contain a highly basic amine usually suffer from poor oral bioavailability in animal models due to low absorption. Substitution of the weakly basic amino-pyridine moiety for the cyclohexylamine found in **30** gave the modestly active **37** (Ki = 12 nM) and led to an improvement in oral bioavailability from <10% to 40-76% in rats and dogs (30). The P3-P2 Phe-Pro group of compound **30** was replaced with the achiral benzylsulfonamide-pyridinone group to give compound **38** (Ki = 4.6 nM) (31). Replacement of the cyclohexyl-amine group of **38** with an amidino-piperidine resulted in a further increase in potency (**39**, Ki = 0.5 nM) but this compound had poor oral bioavailability in animals. Substitution of an amino-pyridine group for the basic N-terminal group in **36** resulted in a loss in potency (**40**, Ki = 11 nM) (32). Addition of a



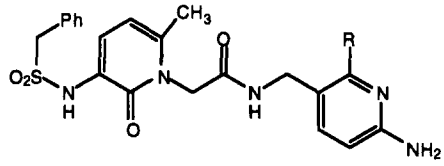
37 Ki = 12 nM



38 Ki = 4.6 nM

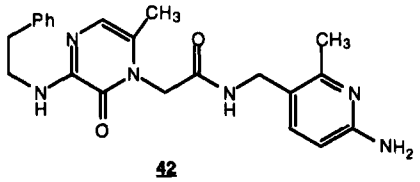


39 Ki = 0.5 nM



40 R = H Ki = 11 nM

41 R = CH_3 Ki = 0.5 nM

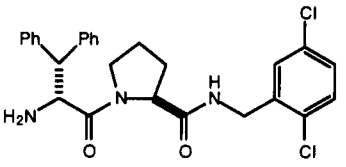


42

methyl group adjacent to the pyridine nitrogen increased potency twenty-fold (**41**, Ki = 0.5 nM) and also gave a similar increase in selectivity towards trypsin. An X-ray structure of **41** in thrombin shows that the aminopyridine group occupies the specificity pocket and that the methyl group interacts with the aliphatic side chain of Val213. Compound **41** was reported to have good efficacy in an animal model of thrombosis and is orally bioavailable in dogs and cynomolgus monkeys. Further modifications of the sulfonamide pyridinone group resulted in the discovery of a novel series of inhibitors which contain a pyrazinone group in the P2 position (**42**) (33).

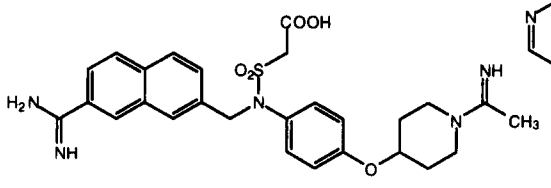
Most compounds presented in this review have contained a charged group in the P1 position which interacts with Asp189 in the specificity pocket. Compound **30** was used as a design template to discover novel P1 groups via a rapid analog resin-

based approach. These efforts resulted in the discovery of several neutral P1 groups which gave potent thrombin inhibitors such as **43** (Ki = 3.0 nM) (34).

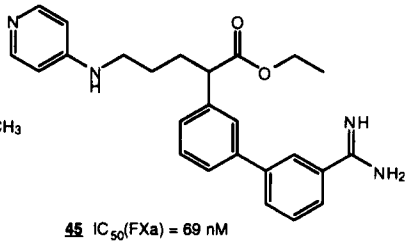


43 Ki = 3.0 nM

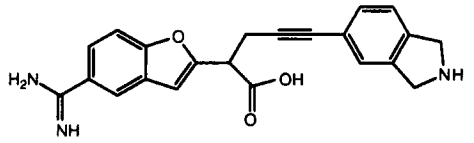
Factor Xa Inhibitors - Factor Xa is a serine protease which cleaves prothrombin to generate thrombin and lies at the crossroads of the extrinsic and intrinsic coagulation pathway. Only a small amount of factor Xa is needed to generate many molecules of thrombin. It has been hypothesized that this would be a better target for controlling thrombogenic events because it would require lower plasma levels of an inhibitor and should have a better therapeutic index for anticoagulation control versus unwanted bleeding events. The development of specific, small molecule factor Xa inhibitors has been less successful than the discovery of thrombin inhibitors and only recently has there been any crystallographic data to help facilitate structure-based design of factor Xa inhibitors (35). Nevertheless, this has become a major focus of many research programs and significant progress is now being reported. Compound **44** was recently disclosed as being a potent (Ki = 1.3 nM) Factor Xa inhibitor with comparable efficacy to argatroban, heparin and dalteparin in a rat arterio-venous shunt model (36). The compounds were also studied for their effects on template bleeding time and **44** had a wider separation between bleeding risk and antithrombotic effects in this model. Other compounds recently disclosed in the patent literature are **45** (IC_{50} = 69 nM), **46** (IC_{50} = 230 nM) and **47** (Ki = 5μM) (37-39). Bis(amidinobenzyl)cycloheptanone **48** can exist with the sidechains either in an all-trans, all-cis, or cis/trans orientation to each other



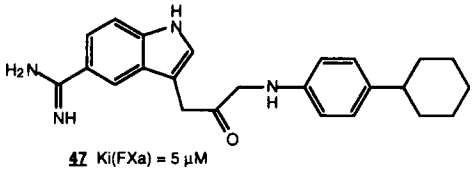
44 YM 60828 Ki(FXa)=1.3 nM



45 IC_{50}(FXa) = 69 nM

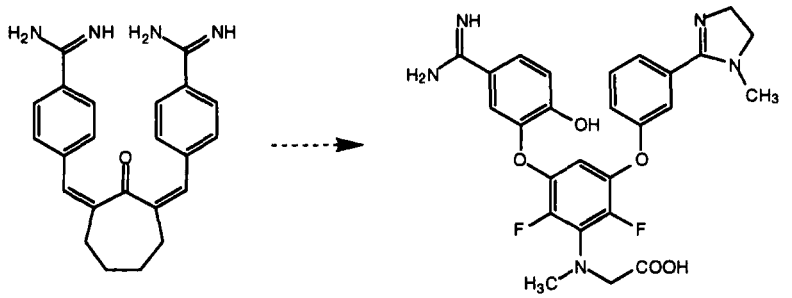


46 IC_{50}(FXa) = 230 nM



47 Ki(FXa) = 5 μM

(40). It was found that the optimal orientation for factor Xa inhibition is when the olefinic groups are in the orientation as shown (41). The bisaylidene-cycloheptanone ring system was replaced by a penta-substituted pyridine ring and the benzamidine groups were systematically modified to afford the optimal inhibitor ZK-807191 (**49**). The compound is potent against factor Xa (Ki = 0.1 nM) and is selective (3200-fold) towards trypsin. Substitution at the 4-position of the pyridine ring leads to selectivity over a number of related serine proteases. The phenol group opposite to the amidine function adds



48 Ki = 0.66 nM (Xa); 33 nM (trypsin)

49 ZK-807191 Ki = 0.1 nM (Xa); 320 nM (trypsin)

potency and selectivity to the molecule and computer modeling studies in a trypsin site (modified to mimic factor Xa) suggest that it binds to the catalytic serine hydroxyl group. It was also reported that 49 is 20% orally bioavailable in dogs and monkeys.

RECENT CLINICAL TRIALS

Clinical Studies with Thrombin Inhibitors - Several articles reviewing the use of thrombin inhibitors in a clinical setting have recently been published (42-46). A clinical study comparing the efficacy of desirudin (hirudin) versus enoxaparin (low molecular-weight heparin) for the prevention of thrombogenic complications after total hip replacement has recently been published (47). Desirudin gave a lower rate of proximal deep-vein thrombosis (DVT) (4.5% vs. 7.5%) and a lower rate of overall DVT (18.4% vs. 25.5%) versus enoxaparin. The combination of enoxaparin plus aspirin was more effective than unfractionated heparin plus aspirin in reducing the incidence of ischemic events in patients with non-Q-wave coronary events (unstable angina or non-Q-wave myocardial events) (48). A study comparing the effects of subcutaneous low molecular-weight heparin versus unfractionated heparin in patients who have a history of pulmonary embolism or venous embolism has recently been published (49). The trial showed that both treatment regimens were equally effective. The preclinical and clinical pharmacology of the small molecule thrombin inhibitor argatroban has been reviewed (50). A study has been published comparing the effects of three different doses of the small molecular-weight inhibitor inogatran versus heparin in patients with unstable coronary artery disease (51). The conclusion was that none of the dosage groups of inogatran had any advantage over heparin in preventing ischemic events. A clinical trial comparing two doses of napsagatran, (a small molecular-weight intravenous thrombin inhibitor) with heparin for the treatment of proximal DVT showed no significant differences in any of the endpoints (52).

Conclusions - The first generation of thrombin inhibitors to reach the clinic were polypeptides derived from natural sources. This was followed by the discovery of small molecule inhibitors which were studied as intravenous agents. We are now at a point where some orally active, small molecule thrombin inhibitors are being introduced into the clinic and results of these studies should be available in the next few years. Finally, a new effort on Factor Xa inhibitors has been initiated and the progress of these inhibitors should not be too far behind those of thrombin inhibitors.

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Chapter 10. Phosphodiesterases 4 Inhibitors

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Introduction - Major advances in our understanding of inflammation at both the biological and molecular level have opened up multiple opportunities for therapeutic intervention in asthma. Some of the more established of these approaches have now been validated clinically, but there is still a long way to go before the mainstay treatments such as steroids and the nonsteroidal anti-inflammatory drugs have significant competition. In the last few years there has been a rapidly increasing research effort into a family of cyclic nucleotide phosphodiesterases (PDE). The PDE4 family which represents a molecular target for new antiasthmatic and anti-inflammatory drugs has attracted much interest (1-3). PDE4 is the predominant form of PDE found in cell-types implicated in chronic inflammatory diseases and, amongst the bone-marrow derived cells only platelets do not express this isozyme (4). The potential benefits of PDE4 inhibitors on inflammatory cell function were initially explored through several in vitro experiments; i.e., these compounds inhibited superoxide generation in human monocytes, eosinophils and neutrophils, mediator release in basophils, macrophages and neutrophils, TNFα-release in monocytes and macrophages (5-12). The anti-inflammatory effects of PDE4 inhibitors, often using rolipram as a standard, were also observed in vivo ; i.e., inhibition of microvascular leakage into the lungs of sensitized guinea-pigs, and reduction of bronchial hyper-reactivity and eosinophilia in cynomolgus monkeys following repeated antigen challenge (13-14). Recent data have shown that PDE4 inhibitors potently suppress TNFα-release from mononuclear phagocytes (15). These observations opened the possibility of using PDE4 inhibitors in the treatment of pathologies associated with over expression and production of TNFα, such as certain auto-immune diseases.

In this chapter we will give an overview of the PDE4 inhibitors which are presently in clinical development. In addition, the main directions that are being employed to develop the next generation of compounds will be discussed ; the obvious therapeutic goal being to optimize on the anti-inflammatory actions of PDE4 inhibitors while formulating strategies to limit their side effects. For this purpose two main areas are actually under investigation ; the first one focuses on the research of the physiological relevance of the

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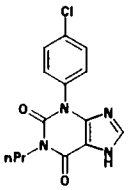
high affinity rolipram binding site and the second one consists of attempts to identify PDE4 subtype selective inhibitors.

PDE4 INHIBITORS UNDER CLINICAL DEVELOPMENT

LAS-31025 (1) (arofylline), a xanthine derivative has been reported to be in phase 3 clinical trials since 1996 (16). Arofylline displayed a PDE4 *in vitro* activity (IC_{50}=5.3μM, PDE4 from guinea-pig heart ventricle) but the affinity of arofylline for adenosine receptors was found similar to the PDE4 IC_{50} (IC_{50} at 3.7μM and 7.2μM for A_1 and A_{2a} respectively)(17). Compared to rolipram (2), arofylline exhibited similar or more potent antiinflammatory activity in the airways : ~57% inhibition at 10mg/kg p.o. for both rolipram and arofylline in the model of guinea-pig airway eosinophil infiltration and 47% and 50% inhibition respectively for rolipram and arofylline in a model of extravasation in sensitized guinea-pig trachea (18). LAS-31025 showed *in vitro* bronchodilatory and *in vivo* antibronchoconstrictor effects (12 and 42 fold more potent than theophylline, respectively). Also, emetogenic potential of arofylline was about 30-fold lower than that of rolipram.

Rolipram (2), from Schering, originally in development as antidepressant therapy (phase 3 in 1991), was dropped for this indication. It is now reported to be in phase 2 for multiple sclerosis (16). The major side-effect observed for rolipram is strong emesis (19). However, rolipram (2) continues to be used as the standard specific PDE4 inhibitor and inhibitor of TNFα-release for evaluation of the mechanism of action in asthma (20-24) and in novel therapeutic indications.

SB-207499 (ariflo) (3) is a PDE4 inhibitor reported in phase 2 for asthma (16). This PDE4 inhibitor retains *in vitro* antiinflammatory activities similar to those of rolipram (2) (histamine-release in basophils, LPS-induced TNFα formation in monocytes, neutrophil degranulation, antigen-driven proliferation and cytokine synthesis). However, it also produces acid secretion from isolated rabbit gastric glands(25). Compared to emesis induced by rolipram, SB-207499 displayed a 100-fold lower emetic potential (ED_{50} at 0.04 and 4.6mg/kg i.v., respectively for rolipram and SB-207499 in dog) leading to a tenfold improved therapeutic potential (26). It is also the first compound to be claimed with a tenfold selectivity for the PDE4D form of the enzyme (27).



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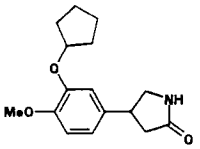

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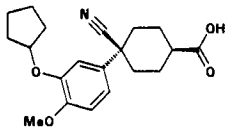
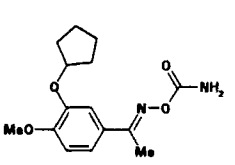


3

WAY-PDA-641 (4) (filaminast), a PDE4 inhibitor reportedly in phase 2 for asthma has an in vitro PDE4 IC_{50} of 0.42μM in dog trachea and is 36-fold selective over PDE3 (16,28). WAY-PDA-641

relaxes respiratory smooth muscle in vitro and in vivo. WAY-PDA-641 is more potent than aminophylline in vitro and in vivo. WAY-127093B (5), another PDE4 inhibitor, (IC_{50}=17nM, dog trachea) has increased selectivity for PDE4 versus PDE3 (IC_{50}>300μM) (29). In guinea-pig after oral administration, WAY-127093B was less potent than rolipram but more potent than aminophylline in inhibiting antigen-induced bronchoconstriction (ED_{50} at 2.9, 0.5 and 43mg/kg p.o., respectively) and was more potent than rolipram (2) in inhibiting antigen-induced airway eosinophilia (ED_{50} at 0.3 and 2.5mg/kg p.o., respectively). Both compounds were equipotent in inhibiting antigen-induced airway eosinophilia in Brown Norway rats (ED_{50} 0.5-0.6mg/kg p.o.).



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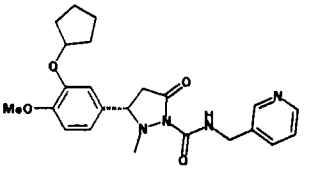
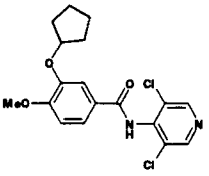


5

The phase 2 development of the selective PDE4 inhibitor RP-73401 (6) (piclamilast) for asthma was discontinued and the compound is now being pursued for rheumatoid arthritis (16,30, 31). PDE4 isolated from various cell types was inhibited by RP-73401 with an IC_{50} of about 1nM and 19,000-fold selectivity against the other PDE isoenzymes. RP-73401 was also reported as a potent and long-acting relaxant of human bronchial muscle in vitro (more potent than rolipram and theophylline) (32). RP-73401 was retained in bronchial tissue for much more longer period of time than rolipram. In clinical studies, RP 73401 did not prevent allergen-induced bronchoconstriction during the early phase reaction in asthmatics (33). The di-N-oxide derivative 7 of RP-73401 showed an IC_{50} at 150nM against PDE4 with a bioavailability of 77%, in rodents, (cf 1% for RP-73401) and an ED_{50} of 1mg/kg when dosed orally in TNFα-inhibition studies (16,34).



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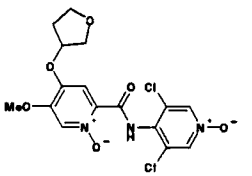
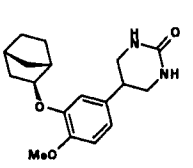


7

CP-80633 (8) (atizoram) showed efficacy in phase 2 clinical trials for atopic dermatitis (35). CP-80633 selectively inhibited PDE4 (human lung, IC_{50}=1.3μM). CP-80633 dose-dependently (3-32mg/kg p.o.) elevated plasma cAMP levels and decreased systemic TNFα-production in mice (ED_{50} at 1.2 and 3.9mg/kg p.o., in control and adrenalectomized mice, respectively) (36). It was also shown to be a potent bronchodilator in a model of histamine-induced bronchoconstriction in anaesthetized guinea-pigs (ED_{50}=10μg/kg i.v.) (37). In a model of antigen-induced cell recruitment in atopic monkeys, CP-80633 (1mg/kg, qid, s.c., 1 hour before antigen-challenge) displayed some antiinflammatory effects by reducing increases

in bronchoalveolar lavage neutrophils and eosinophils (38). CP-353164 (9) was identified as a potent inhibitor of PDE4 (IC_{50}=0.34μM) and TNFα-release in vitro in isolated human monocytes (IC_{50}=0.037μM) and human whole blood (IC_{50}=0.18μM) (39). This compound was equipotent to rolipram (2) and more potent than SB-207499 (3) in vivo in a murine TNFα-production model (68% and 65.2% inhibition at 1mg/kg p.o. and 59.4% inhibition at 10mg/kg p.o. for rolipram (2), CP-353164 (9) and SB-207499 (3), respectively.



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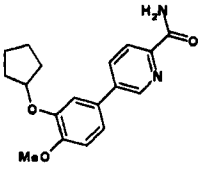
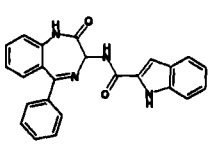


9

D-4418, in phase 1 clinical trials, is reported as being a selective PDE4 inhibitor although the exact structure has not yet been described (40). D-4418 showed a tenfold therapeutic window (difference between efficacious dose in guinea-pigs and emetic dose in ferrets and dogs). In the asthma model of antigen-induced bronchoconstriction/inflammation in sensitized guinea-pigs, D-4418 inhibited the early and late asthmatic reactions (41).

D-22888 (exact structure not reported) displayed a selective in vitro PDE4 activity (IC_{50}=158nM, human leukocytes) (42). TNFα-release was inhibited in 1:5 diluted human whole blood and in rat liver macrophages with IC_{50}s at 8μM and 14μM respectively. The effects of D-22888 were investigated on allergic rhinitis in domestic pigs. D-22888 exhibited an antiallergic effect and dose-dependently inhibited the rhinorrhea. D-22888 is under preclinical evaluation for the treatment of allergic rhinitis and other allergic disorders (16).

A new series of PDE4 inhibitors in a novel benzodiazepine family was recently described (43,44). SAR studies showed the importance of the 5-membered ring fused to the benzodiazepine nucleus for the PDE4 activity : both non-cyclic derivative 10 and 6-membered ring analog 11 lost activity compared to the 5-membered derivative 12 (45). Various modifications were realized on the different aromatic moieties leading to several compounds with PDE4 activity and selectivity over other isozymes PDE1, 3 and 5. From this series, the profile of an orally active selective PDE4 inhibitor, CI-1018 (13) was identified (46). CI-1018 displayed an inhibition of PDE4 isoenzyme from U937 cell line (IC_{50}=1.14μM) with good selectivity over other PDE isoenzymes. This compound inhibited TNFα-production from LPS-stimulated human peripheral blood monocytes (IC_{50}=0.7μM). In vivo, CI-1018 showed good potency and efficacy vs antigen-induced pulmonary eosinophilia in actively sensitized Brown Norway rats (ED_{50}=5.1 mg/kg po) with a duration of action >24 hours. The compound was not emetic at therapeutic doses in several species : while rolipram was emetic at 0.1mg/kg i.p. in ferrets, CI-1018 did not induce any vomiting episodes up to 10mg/kg i.p.. CI-1018 is currently being evaluated in phase 1 clinical trials (16).



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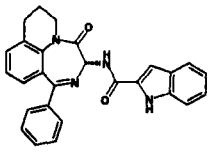

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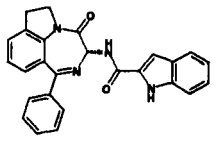

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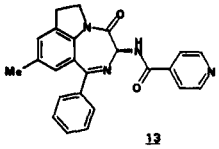
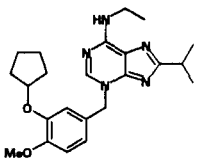


13

V-112294A (**14**), a purine derivative selected for a preclinical evaluation, is a selective PDE4 inhibitor (IC_{50}=200nM, human lung) (16,47). V-112294A inhibited TNFα-release from monocytes (IC_{50}=300nM) and was active in vivo (ED_{50}=8mg/kg p.o.) in a murine model of LPS-stimulated TNFα-release. V-112294A also suppressed antigen-induced accumulation of eosinophils into bronchoalveolar lavage (BAL) in guinea-pigs (85% at 24 hr at 10mg/kg p.o. at -24 hr, -1 hr and +6 hr relative to antigen). In addition, V-112294A induced relaxation of isolated trachea smooth muscle (IC_{50}=17μM) and inhibited antigen-induced bronchoconstriction in allergic guinea-pigs (67% at 10mg/kg p.o.). With a bioavailability >50% in humans and >70% in ferrets, and a plasma half-time in humans greater than 7 hours, V-112294A did not produce any emetic episodes at doses up to 300mg in humans and at 10mg/kg p.o. in ferrets (30,47). In addition, no side effects were observed in CNS, cardiovascular, gastrointestinal or urinary systems at doses up to 75mg/kg p.o..

The structure-activity relationships (SAR) of a series of pyrido[2,3-d]pyrimidin-2(1H)-one derivatives were investigated leading to several compounds with potent and selective PDE4 inhibitory activity. From this series, YM-58997 (**15**), is under preclinical evaluation (16). This selective PDE4 inhibitor (IC_{50}=1.2nM, human peripheral blood cells) was orally active in a model of antigen-induced pulmonary eosinophilia in guinea-pigs (ED_{50}=1mg/kg) (48). In order to develop more potent and orally active compounds, a series of pyrido[2,3-d]pyrimidin-2(1H)-ones derivatives has been investigated. Few compounds were orally active in carrageenan-induced pleurisy model and did not cause emesis up to 100mg/kg p.o. in ferrets (49).



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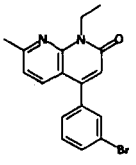
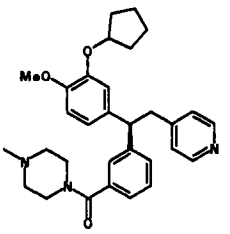


15

The diphenyl pyridyl ethane derivative **16**, is the most promising compound from a new series of CDP-840 **17** analogs (50).

This compound has an in vitro inhibitory PDE4A activity with an IC_{50} at 0.8nM and is under preclinical evaluation (50,51).

The most promising compound **18**, from a series of quinoline-related PDE4 inhibitors, is under investigation (52,53). In a model of inhibition of eosinophil activation, it displayed an IC_{50} of 0.71nM.



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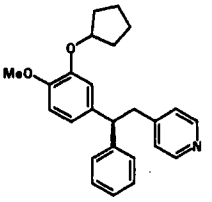

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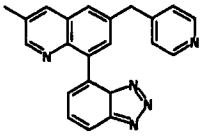
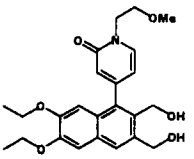


18

A newly generated naphthalene derivative, T-440 (**19**), identified as a selective PDE4 inhibitor (IC_{50}=57nM, guinea-pig lung) raises the intracellular cAMP level of peripheral blood monocytes (PBMCs) in a concentration-dependent manner (54,55). T-440 which is in clinical development, was assessed in various in vivo models (16). This compound administered i.v. to anaesthetized guinea-pigs inhibited histamine-induced bronchoconstriction (ED_{50}=0.08mg/kg) and this effect was closely correlated with T-440 *in vitro* PDE4 activity (56). It inhibited also antigen-induced bronchoconstriction with an ED_{50} of 2.3mg/kg i.v.. T-440 suppressed allergen-induced IL-5 production by peripheral blood mononuclear cells (PBMCs) of atopic asthmatic (IC_{50}=0.039µg/ml) and allergen-induced proliferation of PBMCs (IC_{50}=0.30µg/ml)(10). This activity is probably due to the increase of cAMP which induces reduction of IL-5 production as it has been shown in PBMC from atopic asthmatic subjects (58). T-440 also induced inhibition of IL-2, IL-5 and IL-4 production by concanavalin A activated PBMCs in a concentration-dependent manner (IC_{50} at 0.11, 0.57 and 7.7µg/ml, respectively) (57). T-440 at 10mg/kg p.o. significantly inhibited allergen-induced immediate and late asthmatic reactions in eosinophil infiltration into the airways of guinea-pigs (59). T-440 and rolipram were equipotent in inhibiting ozone-induced airway hyperresponsiveness at 10mg/kg p.o. (60). T-440 reversed histamine-, Ach- and allergen-induced bronchial contraction in the human bronchus by releasing chemical mediators, probably from mast cells (61). The effects were more potent with T-440 than those found with aminophylline (one of the clinical non selective PDE inhibitor). A T-440 derivative **20** coming from a novel series of naphthalene analogs is also under preclinical evaluation for treatment of asthma (62,63). This compound displayed an improved in vitro PDE4 activity (IC_{50}=0.4nM). In guinea-pig, it inhibited histamine-induced bronchoconstriction (ED_{50}=14µg/kg) (16,64).



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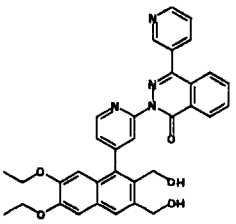
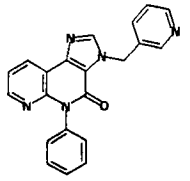


20

KF-19514 (21), a mixed PDE4-PDE1 inhibitor has been studied preclinically in asthma (16), (IC_{50}=0.40μM and 0.27μM for in vitro PDE4 and PDE1 activity) (65). In vitro, KF-19514 produced a more potent relaxation of antigen-induced contraction in guinea-pig tracheal smooth muscle than rolipram (EC_{50}=0.058μM and 0.34μM, respectively) and both induced more potent inhibitory effects on the antigen-induced than on histamine or carbachol-induced bronchoconstriction. In vivo, KF-19514 suppressed antigen-induced bronchoconstriction with a stronger activity than that found in histamine-induced bronchoconstriction (ED_{50} at 0.004 and 0.056mg/kg i.v., respectively in guinea-pigs) suggesting that KF-19514 may have anti-allergic activity and may be considered as a bronchodilator or bronchoprotective agent (66). A complete inhibition of PAF-induced eosinophil infiltration in the airways was observed at 0.5mg/kg p.o., an inhibition of TNFα-production in mice was shown (ED_{50}=0.023mg/kg p.o.) and a total inhibition of antigen-induced hyperreactivity was found at 0.1mg/kg p.o.(65). KF-19514 also inhibited anaphylactic bronchoconstriction after oral administration (ED_{50}=0.2mg/kg) (65). Inhaled KF-19514 (0.0001-0.01%) significantly suppressed PAF-induced airway inflammation and hyperresponsiveness in guinea-pig (IC_{50}=14.8μM/0.00063% against eosinophil accumulation) (67). Moreover, in bronchial smooth muscle from guinea-pig, KF-19514 inhibited in vitro both cholinergic and tachykininergic contraction with a potent inhibitory effect on tachykininergic contraction (IC_{50}=0.49μM) (67).

RS-17597 (22), a PDE4 inhibitor from a series of 8-aryl-1,6-naphthyridinone analogs, is reported to be in preclinical development (16,68). RS-17597 demonstrated a selective PDE4 inhibition (IC_{50}=0.92nM) and in vivo reduced antigen-induced acute bronchoconstriction and cell influx into the lungs of ovalbumin-sensitized guinea-pigs. In vitro, it produced relaxation of guinea-pig tracheal rings contracted with carbachol (16).



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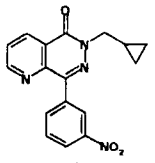
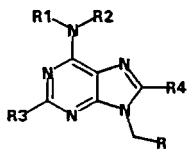


22

NEW PDE4 SERIES

From SAR studies of rolipram-related series, two series of heterocyclic condensed purines led to the identification of new PDE4 selective inhibitors. Some BWA78U analogs 23 exhibited potent

PDE4 activity and selectivity over PDE3 (69). The most important results were from introduction of methyl or trifluoromethyl moieties in position 2 (**24** and **25**, respectively) increasing PDE4 activity (IC_{50} at 0.2 and 0.04μM, respectively) and PDE4 selectivity versus PDE3.



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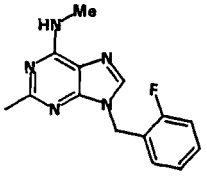

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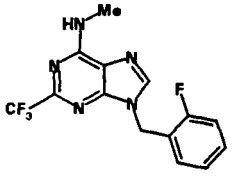
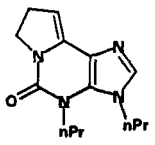


25

A novel series of heterocyclic-condensed purines was designed and synthesized (70). Some of these derivatives, which did not show any adenosine-antagonist activity, had similar or improved PDE4 activity to known PDE4 inhibitors. A correlation between *in vitro* PDE4 activity and *in vitro* tracheal-relaxant activity was found. **26** was the most active and selective PDE4 inhibitor (*in vitro* PDE4 IC_{50}=1.6μM, cerebral cortex) and did not induce emesis in *Suncus murinus* in a range between 10-100mg/kg p.o. while its imidazole analog **27** caused emesis at 10 or 30mg/kg p.o..



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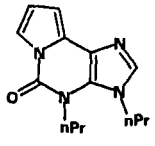
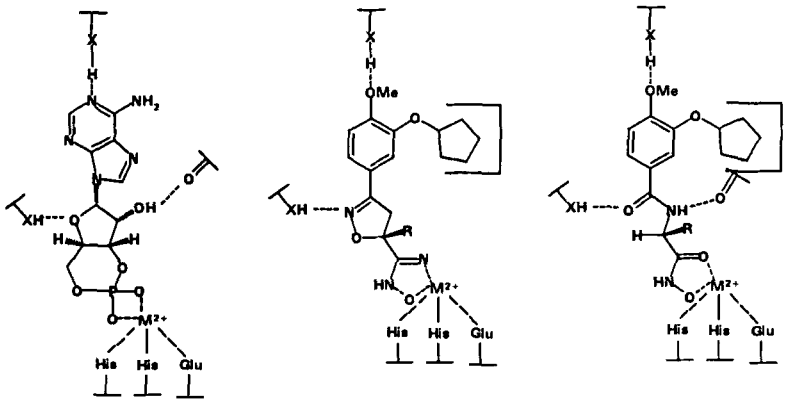


27

To date, a crystal structure of the PDE4 enzyme is not available, limiting our understanding of the catalytic mechanism. Molecular modelling has been performed by exploring the structural requirements for PDE4 inhibition and defining a common pharmacophore model for rolipram-like compounds, xanthine and quinazolidinone derivatives (71).

Various mutational analyses have been performed in order to identify critical amino acids within the central conserved domain of recombinant human PDE4A (72). In parallel, evidence for a zinc binding domain (His-His-Glu) in the active site is supported by SAR studies demonstrating that two novel rolipram-related series with a hydroxamic acid group, a well-known metal chelator, induced a stereospecific interaction with PDE4 (73). By overlapping these two series with cAMP and taking into account that these compounds bind to a metal in the PDE4 active, a model of the PDE4 active site was built. From these series, **28** (R=H) and **29** (R=H) were identified as excellent candidates with nanomolar PDE4 affinities. The best derivative **28** is a potent candidate reported to be under study for cocrystallysation with the enzyme.

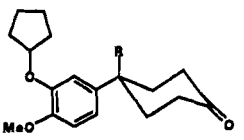


In addition, it has been shown that Zn^{2+} activates purified recombinant human PDE4A with an EC_{50} <1µM (compared to EC_{50} for Mg^{2+} at 100µM). Moreover, titrations of the PDE4A inhibition with Zn^{2+} and Mg^{2+} showed that replacement of Mg^{2+} by Zn^{2+} , as the activating metal ion, led to decreases in PDE4A inhibitor affinities (74).

SELECTIVITY ISSUES

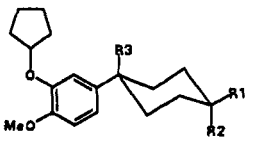
Catalytic activity and HARBS - Historically, a high affinity, stereoselective and saturable [^{3}H]-rolipram binding site in rat brain homogenates has been reported (75). This high affinity rolipram binding site (HARBS) was found as a component of PDE4 (76-78). The functional relevance of the HARBS remained unclear but several research groups have focussed on the hypothesis that inhibitors with potent PDE4 activity and reduced affinity on HARBS would induce relevant antiinflammatory activity with reduced side effects such as emesis, increased gastric secretion and psychotropic activity (19,25,79,80).

In order to achieve this objective, a series of 1,4-cyclohexanecarboxylate derivatives were evaluated by increasing the PDE4 catalytic activity (inhibition of human monocyte-derived PDE4 catalytic activity = LPDE4 activity) over their high affinity on HARBS ([^{3}H]-rolipram binding site in the CNS = HPDE4 activity) (26). From this series, SAR studies of substituents at the benzylic position of cyclohexanone revealed that the nitrile **30** and the acetylenic **31** derivatives were the most promising compounds (IC_{50}s at 44 and 120nM on LPDE4 and HPDE4 respectively for **30** and IC_{50}s at 80 and 300nM on LPDE4 and HPDE4 respectively for **31**).



30 : R = CN

31: R = CCH



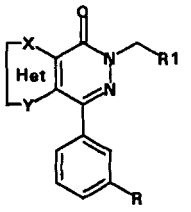
32 : R1 = CO_2H , R2 = H , R3 = H

33 : R1 = H , R2 = CO_2H , R3 = CN

3 : R1 = CO_2H , R2 = H , R3 = CN

The effects of substituents (R1 and R2 of 32, 33, 3) led to the identification of 3, SB-207499, which was equipotent in inhibiting LPDE4 activity (IC_{50}=95nM) and HPDE4 activity (IC_{50}=120nM) : the axial carboxylic isomer 32 was less active (IC_{50}>0.5μM on both HPDE4 and LPDE4). Compared to 3, the analog 32 without the CN substituent induced a reduction in the LPDE4 activity (IC_{50} at 1200 and 120nM for LPDE4 and HPDE4 activity respectively). Compared to R-rolipram, SB-207499 (3) showed an improved therapeutic potential of greater than tenfold.

In parallel, SAR studies of a series of 6-aryl-4,5-heterocyclic-fused pyridazones 34 demonstrated that it was possible to obtain, within this non-related rolipram series, compounds with dissociated PDE4 activity and affinity for HARBS (81).

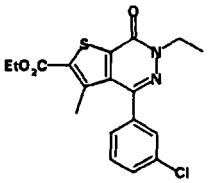


34

Het = thiophene
pyrazole
pyrrole
pyridine

R = H , Cl , NO_2

R1 = Me , Ph



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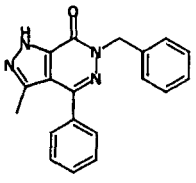

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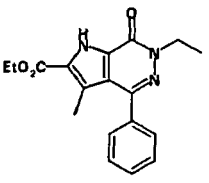

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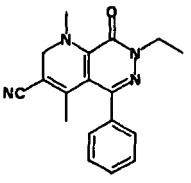


38

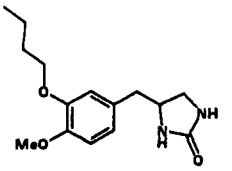
The best compounds (35, 36, 37 and 38) displayed in vitro PDE4 activity in a range between 0.6 to 3.1nM. Furthermore, these PDE4 selective derivatives (over PDE3) presented a lower affinity for the rolipram binding site compared with that found with the nitraquazone-related compound 39 and rolipram.

With the side effects of early PDE4 inhibitors observed, new generation compounds with improved therapeutic windows have now been discovered. To date, two molecular approaches have been

used : one targeting one of the two conformers of PDE4 (82-84) and the other involving design of selective PDE4 subtype inhibitors (82,85).

To date, no correlation has been reported between inhibition of the PDE4 catalytic activity and [^{3}H]-rolipram binding, thus the functional role of the HARBS remains to be defined (86). Also, the functional role of PDE4 inhibitors correlated better with the inhibition of the PDE4 catalytic activity than with the displacement of [^{3}H]-rolipram suggesting that the native PDE4 in human monocytes exist in a low-affinity state (87). Some new information from studies related to the identification of the functional domains of human recombinant PDE4A (88) and PDE4B (78,89) supports the hypothesis that PDE4 exists as two distinct active conformers : one conformer binding rolipram at the catalytic site with a high affinity (HARBS) and the other binding rolipram with a low affinity (83).

PDE4 Subtypes - Recently cDNAs for four members (A,B,C and D) of the human PDE4 family were cloned and the chromosomal localization of the human genes defined (90-92). The four subtypes (A,B,C,D) were identified and characterized and using RT-PCR demonstrated to be differentially expressed between tissues and cells (93-95). Alignment of the amino acid sequence of the human PDE4 subtypes showed three distinct highly conserved domains : the catalytic site and two upstream conserved regions, UCR1 and UCR2, whose function is still unclear (96). Further diversity is also known in this family of enzymes by different splicing and post-translational processing (97). Northern blot analysis of mRNA from different cells and tissues demonstrated the existence of transcripts of different sizes for each of the four PDE4 subtypes (98,99). Detailed analyses of the expression pattern of these isozymes recently appeared and revealed clear differences ; the functional significance of the diverse cellular and tissue distribution of human PDE4 subtypes is still under investigation and remains to be clarified. However it is clear that differential modulation of cAMP levels in different cell types may be feasible, raising the possibility of specific intervention with compounds selective for a single subtype. The low abundance of PDE4 isozymes in the tissues has precluded their purification from natural sources, but the production of recombinant proteins in diverse expression systems offers the opportunity of studying each subtype independently for the identification of distinct biochemical properties that could differentiate them. In a recent study conducted with recombinant PDE4 subtypes expressed in baculovirus the authors demonstrated that although these isozymes have some similar properties (i.e. Mg^{2+} dependency), they also have significant differences with regard to some characteristics (pH dependence, specific activity and sensitivity to drug inhibition) (100). PDE4 subtype selective inhibitors could bring the opportunity to increase the tissue or cell selectivity in a next generation of compounds, decreasing side effects and improving the therapeutic index. To date, no subtype selective inhibitor has been reported. Prototype PDE4 inhibitors do not discriminate between the subtypes (101), however several compounds (rolipram **(2)**, CDP-840 **(17)**, Ro-201724 **(40)** and RP-73401 **(6)** were significantly less potent on PDE4C.

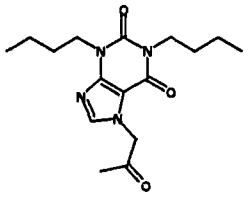


40

OTHER THERAPEUTIC INDICATIONS THAN ASTHMA

cAMP is an ubiquitous second messenger that is involved in various biological responses. Phosphodiesterase 4 inhibitors, by increasing intracellular cAMP levels, lead to the inhibition of inflammatory cell activation. In particular, PDE4 inhibition decreases cell proliferation, adhesion and chemotaxis and inhibits inflammatory mediator or cytokine release such as TNFα by various inflammatory cells (102,103). As many pathologies are due to the dysregulation of the inflammatory response or to immune disorders, there is an increased interest in the use of PDE4 inhibitors in the treatment of diseases other than asthma.

Atopic dermatitis (AD) - AD is the most common chronic skin disease in young children and it is frequently associated with asthma and allergies, suggesting that PDE4 inhibition may offer a new therapeutic approach directed at correction of the immune dysfunction associated with this disease (104). A recent study showed that the type 4 phosphodiesterase inhibitors rolipram (2), Ro-201724 (40) and denbufylline (41) produced a concentration-related inhibition of proliferation of human peripheral blood mononuclear cells (HPBM) from normal and subjects with AD. In addition, this study indicated that the proliferative response of HPBM from AD subjects was more sensitive to PDE4 inhibition than the proliferation of HPBM from normals (11).

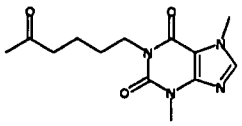


41

Arthritis - Recent studies demonstrated the essential role of tumor necrosis factor α in the pathogenesis of many infectious and inflammatory diseases one of these being arthritis (106). TNFα levels are known to increase both in plasma and synovial fluid of patients suffering from rheumatoid arthritis (107). In a model of collagen II-induced arthritis in rats, very similar to human rheumatoid arthritis, rolipram (2) given subcutaneously 2mg/kg twice daily for five days before the onset of arthritis significantly delayed the appearance of the symptoms (108). However, after the cessation of the treatment, these animals started to develop an arthritis that demonstrated all the characteristics of the disease in untreated animals and reached the same arthritis top score as the control group. In the same study, rolipram (3mg/kg) given subcutaneously twice daily at the

time point when arthritis was apparent, drastically changed the development of the disease : progression of severity was halted and even after the cessation of treatment, the arthritis score did not reach the levels observed in untreated animals. The investigators were also able to demonstrate a strong down-regulation of TNFα and IFNγ mRNA expression in regional lymph nodes suggesting that the major effect of rolipram is exerted in the effector phase of the inflammatory process (109).

Multiple sclerosis - Increasing evidence show that PDE4 inhibitors may be useful in the treatment of multiple sclerosis (110).It has recently been demonstrated that rolipram **(2)** suppresses the clinical manifestations of acute experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and inhibits the production of TNFα (111-113). More recent studies show that continuous PDE4 inhibition by rolipram (3x6.25mg/kg/day) reduced the clinical signs of EAE during both the initial episode of disease and subsequent relapses. Rolipram also reduced gene expression of several TH1 proinflammatory cytokines (including TNFα and IL2) in the CNS (114). Pentoxifylline **(42)** (PTX), a mixed PDE3-4 inhibitor was investigated in a model of experimental autoimmune neuritis (EAN) in rats immunized with peripheral nerve myelin containing neuritogenic peptide SP26. At 200mg/kg/day, PTX **(42)** significantly suppressed clinical EAN, weight loss and T cell proliferation (115).



42

Septic shock - In a model of endotoxin-induced acute renal failure in rats, the selective PDE4 inhibitor Ro-201724, given as a posttreatment (10μg/kg/mn), significantly increased urinary cAMP excretion, markedly attenuated endotoxin-induced increases in renal vascular resistance and decreases in renal blood flow and glomerular filtration rate. Ro-201724 **(40)** also improved survival rates for endotoxin-treated rats (116). Pentoxifylline **(42)** has been studied in patients suffering from septic shock. Twenty-four patients fulfilling the criteria of septic shock were included in this study : 12 received PTX at 1mg/kg/hr over 24hr, 12 served as a control group. After 24h, TNFα levels were significantly lower in the therapy group, while IL-6 showed a significant increase. Nevertheless, inhibiting a single mediator in severe septic shock cannot inhibit completely the inflammatory response (117). In another study, pretreatment with PTX (5 to 50 mg/kg i.p. 3x) or the selective PDE4 inhibitors rolipram (10 to 30mg/kg i.p. 3x) and denbufylline **(41)** (0.1 to 3mg/kg i.p. 3x) reduced LPS-induced bowel erythrocyte extravasation in rats and denbufylline was 100 fold more potent than and PTX in inhibiting LPS induced mesenteric blood flow fall, without affecting renal blood flow or cardiac index. This suggests that potent and selective PDE4 inhibitors

with a good safety profile could be a promising therapy in this field (118).

Inflammatory Bowel Diseases - Crohn's disease - In recent years, an important role for TNFα as a pivotal pro-inflammatory mediator in Crohn's disease has emerged and this has resulted in the development of several therapeutic strategies that target TNFα transcription by increasing intracellular cAMP concentrations (119). PTX (42) was studied in vitro on PBMCs and organ cultures of inflamed mucosa from patients suffering from IBD. In this study, PTX inhibited TNFα-release from PBMCs by 50% at a concentration of 25μg/ml but IL-6 and IL-8 concentrations were not decreased suggesting that PTX may not be potent enough (120). The same compound was studied in patients suffering from Crohn's disease but failed to show clinical efficacy (121). In an experimental model of colitis using intracolonic injection of trinitrobenzene sulphonic acid (TNBS) in rats, PTX given intracolonically at 100mg/kg 24 and 48 hours following TNBS treatment, significantly reduced the gross morphology damage scores compared to animals receiving saline, but did not decrease TNBS-induced elevation of colonic collagen contents (122). Further studies are required with more potent and selective PDE4 inhibitors to discover whether there will be a real therapeutic effect of PDE4 inhibitors in IBD.

Liver injury - TNFα causes hepatic failure in humans, a phenomenom that has been widely studied in various experimental animal models. In an acute model of T-cell mediated hepatic failure, rolipram (2) (0.1 to 10mg/kg i.p. 30mn before the challenge either with Concanavalin A (Con A) or Staphylococcal enterotoxin B (SEB) was shown to significantly reduce plasma TNFα and INFγ concentrations whereas it significantly elevated IL-10 levels (123). Rolipram also suppressed Con A-induced IL-4 release. The plasma activities of liver specific enzymes ALT, AST and SDH were also assessed as their increase indicates massive liver cell destruction. Pretreatment of naive mice receiving Con A or galactosamin-sensitized mice receiving galactosamin/SEB with rolipram (0.1 to 10mg/kg i.p.) dose-dependently inhibited plasma enzyme activities. These results suggest that PDE4 inhibitors may be of considerable interest in the treatment of T cell disorders such as liver failure.

Pulmonary hypertension - The activity of phosphodiesterases which hydrolyze vasodilatory second messengers (cAMP and cGMP) may be increased by hypoxia-induced pulmonary hypertension (HPH). Tested in a model of isolated pulmonary artery rings from normal rats and rats with HPH, rolipram was shown to potentiate the relaxant activities of isoproterenol and forskolin. In this study, the same effect was observed with milrinone (a selective PDE3 inhibitor) suggesting that inhibition of PDE3 and PDE4 activity can significantly improve pulmonary artery relaxation in HPH (124).

Bone loss disease : Denbufylline (41) was examined on bone loss in Walker 256/S-bearing rats and on mineralized nodule formation and osteoclast-like cell formation in bone marrow culture systems. Serial oral administrations of denbufylline inhibited the decrease in the bone mineral density of femurs from Walker 256/S-bearing rats, restored the bone mass and the number of osteoclasts and osteoblasts per trabecular surface in the femur metaphysis. It

also increased the number of mineralized nodules and decreased the number of osteoclast-like cells in the in vitro bone marrow culture system. These effects seem to be specific for PDE4 inhibition and are mimicked by dibutyryl cAMP suggesting that the PDE4 isoenzyme may play an important role in bone turnover through cAMP and that its inhibitors are candidates for therapeutic drugs in diseases involving bone loss (125).

Conclusions - One of the most intensively studied therapeutic targets currently of interest to the pharmaceutical industry is the inhibition of type 4 phosphodiesterase enzymes. PDE4 inhibitors are very attractive anti-asthmatic drugs because of their therapeutic profile consisting of a broad spectrum of anti-inflammatory activities coupled with additional bronchodilatory and neuromodulatory actions. With such a plethora of new, structurally different and potent PDE4 inhibitors, presented in this review, it is reasonable to expect that in a near this class of antiinflammatory agents will find utility in asthma and possibly other inflammatory diseases. Opportunities exist to increase the therapeutic window of safety of the first generation inhibitors. It has yet to be determined whether the second generation of PDE4 inhibitors will overcome emesis and other GI side effects to allow the full therapeutic potential of these compounds to be realized. Treatment of other inflammatory and immune diseases such as rheumatoid arthritis and atopic dermatitis is also a significant opportunity for this class of agents. Only time will tell whether significant advantages will be offered by this new alternative approach of PDE4 inhibition compared to existing medication in the treatment of asthma, but early clinical data in some instances clearly suggests an optimistic forecast. It is also likely that rapid advances in genomics will lead to an increasing array of novel targets, ultimately providing hope for more effective anti-inflammatory treatments.

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Chapter 11. Adenosine

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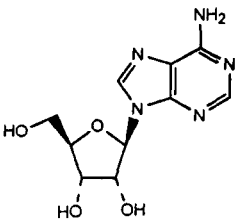
INTRODUCTION

Adenosine **1** is essential for the proper functioning of every cell in the body. It is a component or precursor to a component of RNA and DNA, and in its native form, or in various phosphorylated states, acts as a neurotransmitter at the P1 and P2 purine receptors. The intra- and extra-cellular levels of adenosine are tightly regulated through its production from ADP and ATP, transport through specific nucleoside transporters, and catabolism (1). Levels rise sharply during periods of stress and trauma and thus it has been considered as an endogenous protective agent and many of its functions relate to such a role. Adenosine and its derivatives exert their actions through two classes of receptors called P1 and P2. The development of the P2 receptors which recognize phosphorylated adenosine derivatives has been slow and will not be discussed in this review. Progress around the P2 receptors has been recently summarized (2,3). In contrast, the adenosine P1 receptors have received much more attention. There are four receptors in this family which are distinguished by, among other factors, having a high (A1, A2a) or low (A2b, A3) affinity for adenosine (4-7). The physiological reason for this difference is not well understood, but it is likely that the latter receptors would only be activated during times of extreme stress, trauma or ischemia. They are all prototypical G-protein coupled receptors containing seven transmembrane-spanning domains. The A3 receptor is the newest of this family, being identified in 1991 (8,9). This chapter will summarize the therapeutic potential and recent SAR and pharmacology of adenosine and its receptor agonists and antagonists.



1 Adenosine

THERAPEUTIC OPPORTUNITIES

The past several years have seen unprecedented growth in the therapeutic potential of adenosine and adenosine derivatives (10-14). This development is due in large part to the availability of selective agonists and antagonists for most of the adenosine receptors. The exception to this progress is the A2b receptor, for which selective ligands remain elusive. Despite the large body of data linking adenosine with certain disease states, clinical development of modulators of adenosine activity has been slow. To date, the only marketed agent in this area is adenosine itself. The reasons for this slow development are manifold. Adenosine receptors are widely distributed throughout the body. This fact coupled with the wide range of activity these receptors regulate make a poor combination for drug therapy. In addition, no non-nucleoside agonists have been reported and although some oral activity has been demonstrated for nucleoside derivatives, they generally suffer from both low bioavailability and short half-lives (15). Some adenosine agonists also show tendency for tachyphylaxis which would likely prevent their use for chronic conditions (16). Adenosine antagonists do not suffer from such limitations. The structures of these

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inhibitors are also more varied and not surprisingly it is this class that has progressed further clinically. There is also some species differences between the receptors particularly regarding the A3 subtype (17-19). In fact, there have been suggestions that the A3 receptors from the rat and human may not be equivalent. This difference is significant in two ways. First, the rat receptor is most commonly used in radioligand binding assays thus leaving questions about a compound's selectivity in other species. Secondly, rat is often used for *in vivo* studies, making connections between rat and humans from such studies questionable.

With the above restrictions accepted there are still a number of potential indications where adenosine modulation is a viable approach. These include the use of agonists in acute settings with acceptable routes of administration (e.g. topical, inhalation or parenteral) or the use of antagonists which have fewer pharmacokinetic issues. Detailed below are the therapeutic areas which have the greatest potential for pharmaceutical intervention with an adenosine related drug.

Ischemia- Besides inducing hemodynamic effects, adenosine is probably best known for its ability to protect organs, including the heart and brain, from ischemic injury. The involvement of adenosine in this preconditioning phenomenon follows from the early work on mechanical preconditioning (20). Brief periods of mechanically induced ischemia protect tissues from a subsequent longer period of ischemia. During these short preconditioning episodes it was discovered that adenosine levels rose sharply. Adenosine could be shown to induce a number of changes beneficial to a hypoxic cell including hypothermia, bradycardia and preservation of cellular ATP levels (21-23). These effects were attributed to activation of the A1 receptor which was supported by the fact that selective A1 agonists were also protective (24,25). Although the hemodynamic effects induced by the A1 agonists have been shown not to play a major role in cardioprotection, they present a major hurdle for the development of such an agent. More recently, selective agonists at the A3 receptor have shown promise as cardio- and cerebro-protective agents without causing changes in blood pressure or heart rate in animal models (26-28). Because adenosine agonists are involved here, disease states requiring short term treatment and non-oral routes of delivery are best suited. Such possibilities include acute coronary syndrome, acute stroke, and perioperative ischemic injury (29). Adenosine itself has demonstrated positive effects when given prior to PTCA and is now undergoing further clinical trials as a cardioprotective agent (30,31).

Acadesine, an adenosine modulating agent with a complex mode of action, has been extensively studied clinically for the reduction of cardiac events following coronary artery bypass surgery. However, five separate clinical trials failed to show a statistically significant effect and thus the compound was dropped from development. However, meta analysis of the five studies did show a positive effect on early cardiac death, MI, and combined adverse cardiovascular events (32).

Asthma- The involvement of adenosine in asthma has the added weight of human studies (33-35). Adenosine causes bronchoconstriction, but only after challenge with an allergen and only in asthmatics. The effect could be blocked with theophylline, a weak non-selective adenosine antagonist. More recent studies have implicated the A1, A2a, and A3 receptors in asthma (36-39). The system is complicated by the apparent priming necessary to see a biological response. However, asthma is a disease well suited for the limitations of adenosine therapy because the doses would be administered topically and in aerosolized form. The true role of adenosine receptors in asthma may only be satisfactorily resolved in the clinic.

Glaucoma- The evidence suggesting a role for adenosine in the reduction of intra-ocular pressure has recently been reviewed (40,41). This effect appears to be mediated through A1 receptor agonism. Importantly, no desensitization was observed in the rabbit eye after five days of exposure to CPA, a selective A1 agonist. This pressure lowering has been reported to be a result of reduced aqueous humor inflow but the molecular mechanism has not yet been fully elucidated (41).

Pain- There exists strong evidence, including clinical experience, that adenosine possesses antinociceptive properties (42). This appears to be, at least in part, a local effect perhaps mediated by the A1 receptor. Used clinically, adenosine has demonstrated activity against ischemic pain, post-operative pain and peripheral neuropathic pain (43-46). Adenosine and adenosine analogs are now being studied clinically using local, intrathecal administration for pain control.

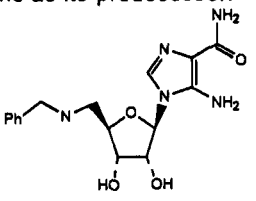
Central Nervous System- Adenosine receptors are widely distributed in the central nervous system (CNS) where adenosine acts to depress neuronal activity. Not surprisingly, adenosine antagonists, caffeine being the most well known agent in this class, are CNS stimulants. As an endogenous depressant, adenosine is implicated as a sleep inducing agent. Adenosine levels in the brain rise sharply upon prolonged wakefulness (47). In addition, a selective A2a agonist increased slow-wave sleep and paradoxical sleep in rats in a dose dependent manner (48). This sleep inducing effect could be attenuated by the administration of the A2a antagonist KF-17837. Interestingly, KF-17837 also reduced the sleep inducing effect of PGD2, another proposed sleep-promoting agent, suggesting its ultimate action on the adenosine receptor system.

In the brain, adenosine is released in high concentrations during cerebral ischemia and other stress and trauma situations. In this role, adenosine is considered to be an endogenous anticonvulsant/neuroprotectant (49,50). In fact, adenosine modulators have shown both pro and anticonvulsant properties and have demonstrated the ability to positively attenuate the anticonvulsant effects of other agents. Adenosine agonists are also neuroprotectants during periods of cerebral ischemia through the same mechanisms as the cardioprotective effects (51). However, therapeutic intervention for CNS ischemia has the added challenge of discovering compounds which cross the blood brain barrier.

Perhaps the most intriguing adenosine connection in the CNS is its relationship to the striatal dopaminergic system (52). Stimulation of the central A2a receptors in this region results in an inhibitory response on dopamine neurons while A2a antagonists increase dopaminergic activity. These observations have led to the theory that A2a agonists may be novel atypical antipsycotic agents, whereas A2a antagonists may have therapeutic benefit in Parkinson's disease (53). Compounds for the latter indication are already in clinical development. However, their use may be limited to early Parkinsonism since this modality relies on the ever decreasing number of dopamine producing neurons to be effective. The development of A2a agonists for schizophrenia seems unlikely at this time due to drug delivery/pharmacokinetic issues as well as the likelihood of serious hemodynamic side-effects.

Miscellaneous- In addition to the indications listed here, adenosine has been implicated in a number of other disease states. Some of these include cancer (54,55), inflammation (56-59), fever (60-62), diabetes (63-65), and wound healing (66).

Adenosine Compounds in Clinical Development- This section is devoted to adenosine compounds currently undergoing clinical investigation. A number of other agents have been dropped from development in recent years, mostly due to unacceptable side-effects. GP-1-531(2) is an adenosine regulating agent in the same class as acadesine, which was discussed earlier (67). It is reportedly in phase II trials for the same indications as its predecessor.



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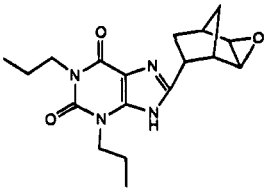

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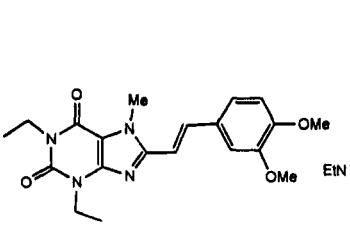
CVT-124 (3) is a selective A1 antagonist with diuretic activity. It has been initially targeted for the treatment of edema in hospitalized congestive heart failure patients and is now in phase II trials. In healthy volunteers, CVT-124 increased sodium and water excretion while preserving potassium levels (68). KW-6002 (4) is a xanthine derived selective antagonist at the A2a receptor (69). The compound is currently in phase II clinical trials for the treatment of Parkinson's disease as a monotherapy or in combination with L-dopa. RPR-100579 (5) is a modified nucleoside with activity at both the rat A1 (Ki = 4nM) and A2a (Ki = 47 nM) receptors (70). It contains a carbocyclic ring in place of the ribose and it lacks the N1 nitrogen on the purine. It was last reported in phase I trials for the prevention of myocardial ischemic injury. GR-79236 (6) is a selective A1 agonist with a long development history. It failed in earlier trials as a cardiostimulant and for the treatment for diabetes. It has reentered early clinical trials for the management of pain.



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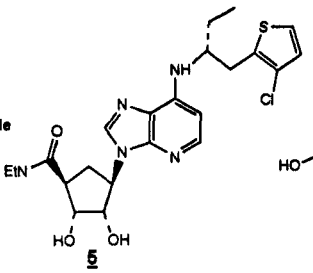

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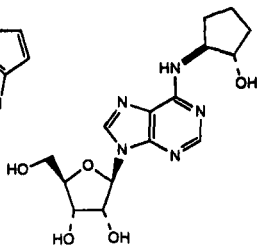


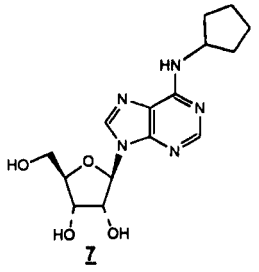
6

MEDICINAL CHEMISTRY

Studies aimed at elucidating the role of adenosine in human disease have been aided greatly by the availability of selective agonists and antagonists at the adenosine receptors. There is a clear correlation between the presence of these tools and information regarding the functions of a particular receptor in vitro and in vivo. The A1 receptor has been the most widely studied in keeping with the long-standing availability of selective ligands. In contrast, agonists and antagonists for the A3 receptor have only recently been described, and accordingly, the pharmacological role of the receptor is only now being unraveled. Also, the paucity of biological data surrounding the A2b receptor can be understood in light of the present poor state of SAR development of this receptor (72). Several recent reviews have described the progress of the medicinal chemistry of the adenosine receptors in some detail (73-75). Only the highlights of this work as well as more recent contributions will be described here. Unfortunately, data for the human receptors is scarce, and unless otherwise stated, the reported activities are from the rat.

A1 RECEPTORS

Agonists- Selective agonists at the A1 receptor can be obtained simply by the addition of a N6 substituent, particularly an α-branched substituent. CPA (7) is one of the most well-known agents potent and selective for the A1 receptor. Its human Ki is 1 nM for A1 and it is almost 200 fold selective against the other adenosine receptors. SDZ WAG-994 (8) contains the added element of 2'-O methylation. Although this modification resulted in some loss of potency, the compound has shown good oral bioavailability in the rhesus monkey (6).



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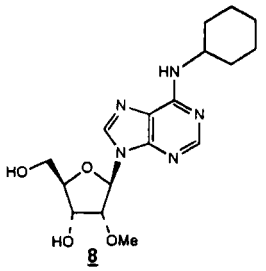
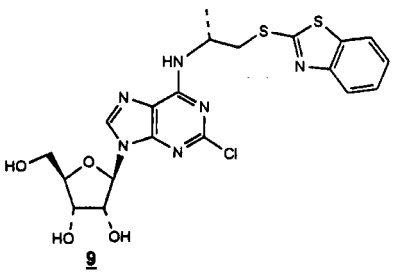


8

NNC 21-0136 (9) is reported to be a CNS selective agonist with minimal cardiovascular effects (76). Compound 10 was one of a series of compounds designed to be partial agonist at the A1 receptor in an effort to reduce cardiovascular side effects and receptor desensitization (77). The exemplified compound had only 40% the intrinsic activity of adenosine.



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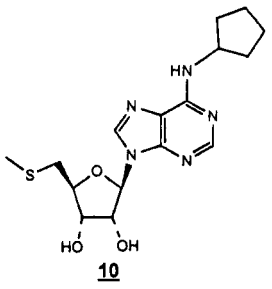
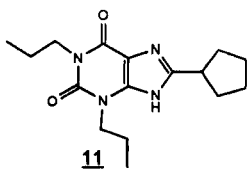


10

Antagonists- Many classes of A1 antagonists have been reported (75). The earliest examples were from the xanthine class typified by DPCPX (11) and KW-3902 (12). Since then, a number of other heterocyclic series have been discovered that show good potency and selectivity at the A1 receptor (13-15) (78-80).



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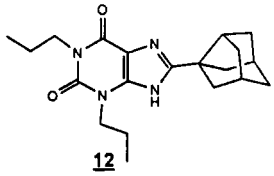

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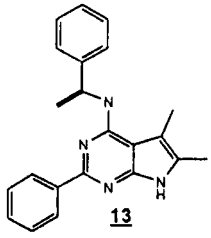
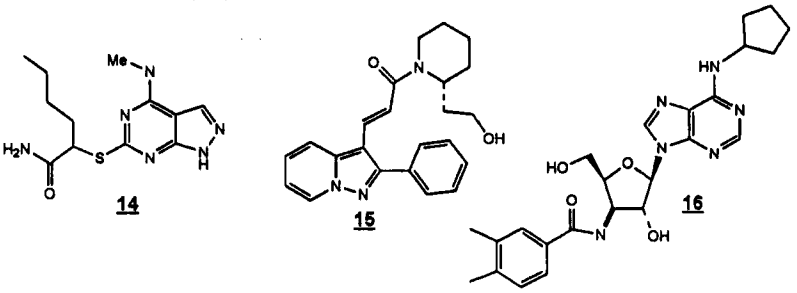


13

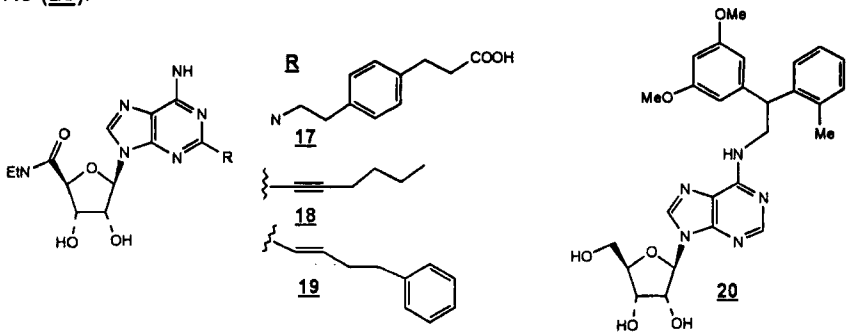
Compound 13 for example, has a Ki for the rat A1 receptor of 7 nM and is more than 4000 fold selective (78). Modification at the C3' position of the A1 agonist CPA led to

the nucleoside based antagonist **16** which was the most potent of the series with a Ki of around 20 nM in the rat (81).

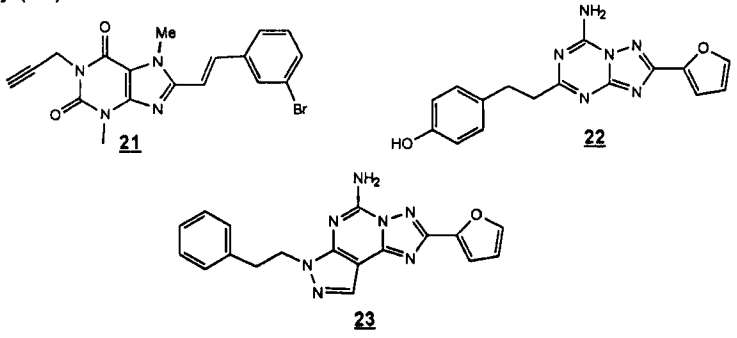


A2a RECEPTOR

Agonists- Almost universally, A2a agonists can be distinguished by substitution at C2. This characteristic is evident in CGS 21680 (**17**), **18** and **19** (82). However, moderately A2a selective agonists have also been discovered with only modification at N6 (**20**).



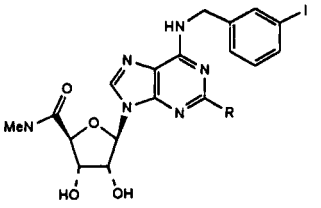
Antagonists- As with the A1 receptor, A2a antagonists have covered a wide variety of heterocyclic core structures. A number of xanthine based analogs have been described, such as BS-DMPX **21** which shows good potency and selectivity at the A2a receptor. A recently described agent, **23**, has a Ki of 2.4 nM and 200 fold selectivity (83).



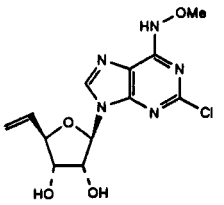
A3 RECEPTOR

Agonists- Not long after its discovery, SAR around this receptor began to develop. For optimal activity it was found that the methyl amide at C5' and benzyl substituents at N6 were preferred. From extensive SAR, IB-MECA (**24**) and Cl-IB-MECA (**25**) were

identified as potent and selective agonists in the rat (84-87). However, as discussed earlier, the rat and human A3 receptors are non-homologous. This difference is manifested in the pharmacology since compound **24** is 50 fold selective in the rat (vs. A1), but only 10 fold selective in human (88). The C5' ethenyl derivative **26** was reported to be 50 fold selective for the human A3 receptor (vs. A1) and possessing an A3 Ki of 20 nM (89).

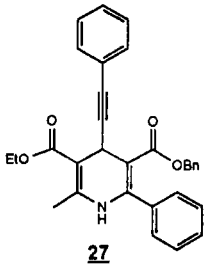


24 R = H
25 R = Cl



26

Antagonists- Only recently have antagonists, selective for the A3 receptor, been described. Several series now exist with quite diverse structures. Modification to a weak A1 antagonist lead resulted in the dihydropyridine derivative MRS1191 (**27**) with a human A3 receptor Ki of 31 nM (90). A similar approach, this time starting from an A2a ligand, yielded the highly potent (human Ki = 0.65 nM) analog MRS1220 (**28**) (91). Two recent patent applications described novel A3 antagonists, such as L268605 (**29**) and **30** (92, 93).



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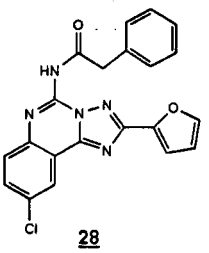

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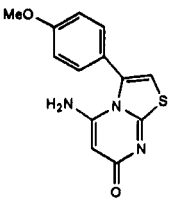

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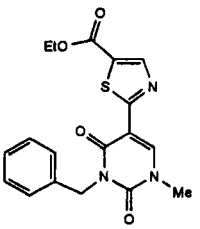
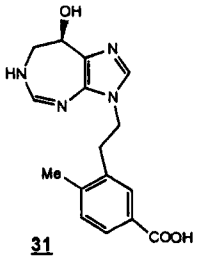


30

Indirect Adenosine Modulators- In addition to the direct acting agonists and antagonists described above, several other indirect methods of adenosine potentiation have been investigated. In general, these agents inhibit the catabolism or uptake of adenosine thereby leading to increased extra-cellular levels of the nucleoside. One potential benefit of these approaches is that they may provide a more site-specific therapy, preserving the beneficial effects of elevated adenosine levels only at the region of potential injury. Three main mechanisms have been targeted for intervention: adenosine kinase, adenosine deaminase and adenosine transport inhibitors. Compounds from each of these three classes have shown efficacy in animal models of some of the same diseases for which adenosine agonists have shown benefit (94-101).



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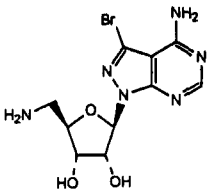
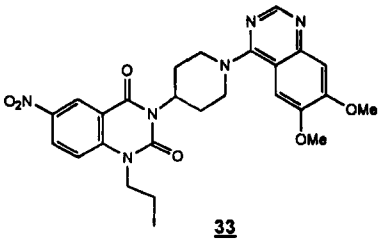


32



33

The coformycin analog **31** represents one class of adenosine deaminase inhibitors and has a Ki for this enzyme from porcine heart of 97 nM (102). A series of C5' amino nucleosides, such as **32** are being developed as adenosine kinase inhibitors (103). GP-1-515 (**32**) inhibited the enzyme from human cardiac tissue with an IC50 value of 4nM. Compound **33** was shown to be a potent (IC_{50} = 62 nM) inhibitor of adenosine uptake into human erythrocytes (104).

SUMMARY

Research focused on methods of adenosine modulation is at an all time high. Medicinal chemistry has provided selective agonists and antagonists for three of the four presently known adenosine receptors. There is an ever increasing understanding of the role of adenosine in disease as well a growth in the chemical arsenal available for pharmaceutical intervention. It appears to only be a matter of time before a selective adenosine modulator joins adenosine as an approved agent for the treatment of human disease.

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SECTION III. CANCER AND INFECTIOUS DISEASES

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Chapter 12. Antimicrobial Potentiation Approaches: Targets and Inhibitors

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Introduction - Over the past several years, the emergence of organisms resistant to nearly all of the major classes of antimicrobial agents has become a serious public health concern (1-3). As pointed out in a number of recent reviews (4,5), antimicrobial resistance is a worldwide phenomenon which is having a significant impact on therapeutic strategies to treat a variety of bacterial, fungal and protozoal infections.

The emergence of bacterial resistance to a variety of antimicrobial agents such as the β-lactam antibiotics, aminoglycosides, macrolides, glycopeptides and quinolones is quite serious (1). Several of the organisms causing the most significant problems include staphylococcus, enterococcus and streptococcus (6,7). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, for example, comprise approximately 40% of all *S. aureus* hospital isolates in the United States (8). Since the emergence of MRSA, vancomycin has been the only uniformly effective agent available to treat serious staphylococcus infections (4); however, reports in 1997 of vancomycin-intermediate resistant *S. aureus* (VISA) clinical isolates in Japan and the United States have caused considerable concern among health care providers (9). More troublesome is that patterns of resistance can be magnified by transfer of the genetic information among species (10), and recent studies suggest that resistant bacteria are persistent in nature due to the stability of resistance genes and transfer elements (11). Antimicrobial resistance is not strictly limited to bacteria. For example, reports of resistance to the antifungal agents fluconazole and ketoconazole have begun to appear (2,12), and the isolation of strains of *Plasmodium falciparum* (the etiologic agent responsible for malaria) which are resistant to several drugs such as chloroquine leave few therapeutic options for control of this infection (3,13).

There are three basic strategies that microorganisms employ in response to the pressure of antimicrobial therapy (14,15). They include i) drug inactivation (16) ii) target modification (17) and iii) alteration in target accessibility primarily through increased efflux of drug (18). Resistance to the penicillins and cephalosporins, for example, frequently results from inactivation of these compounds by β-lactamases, enzymes which catalyze the hydrolysis of the β-lactam nucleus (4). One of the most successful approaches in overcoming the problem of β-lactamases has involved the development of β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (19). These compounds have little intrinsic antibacterial activity but rather potentiate the activity of the penicillin or cephalosporin by protecting the β-lactam ring from inactivation.

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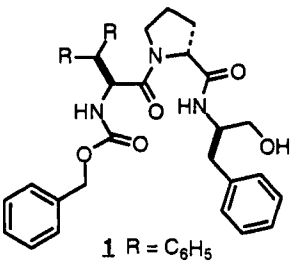
The successful strategy of using a β-lactamase inhibitor to potentiate the activity of β-lactams may be applied to overcoming other microbial resistance mechanisms (14,15). For example, inhibition of an efflux pump would potentiate the activity of a known antimicrobial agent and confer enhanced susceptibility to a particular organism (20). Indeed, an effective approach in tackling the growing problem of antimicrobial resistance may include not just the discovery of new compounds and elucidation of novel targets but the development of potentiators of known antimicrobial agents as well (21). The following chapter will examine the opportunities available and strategies undertaken to overcome several microbial mechanisms of resistance by extending the potentiator approach beyond the β-lactamase arena.

INHIBITION OF METHICILLIN RESISTANCE IN STAPHYLOCOCCI

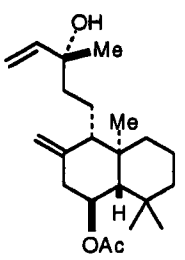
MRSA was first described in the early 1960's and has since become one of the major nosocomial infections in the hospital setting (22). The prevalence of MRSA throughout the world continues to increase with MRSA strains being reported in countries such as Japan, Argentina, Spain and Canada (4,22). Nearly all strains of MRSA share a common feature of carrying the *mecA* gene which encodes for a low affinity penicillin-binding protein, PBP2a (22-24). Production of this protein confers an intrinsic resistance to all β-lactams. Expression of the *mecA* gene depends on two regulatory systems, *mecR1-mecI* and *blaI-blaR1* (25) as well as several auxiliary genes designated *fem* (factors essential for the expression of methicillin resistance) (26,27).

From a drug discovery standpoint, a logical approach to overcoming methicillin resistance would involve the inhibition of any of the resistance factors described above by the use of a small molecule inhibitor in combination with a β-lactam (4). Several reports describing such an approach have recently appeared. For example, the unnatural tripeptide, LY301621 (**1**), potentiates the activity of methicillin against MRSA and acts as a relatively weak, stand-alone inhibitor of bacterial growth (28). SAR studies highlight the importance of the topology of **1** on potentiation activity (29).



1 R = C_6H_5

A more directed approach involved screening for compounds which displace the binding of radiolabelled penicillin to PBP2a (30). The screen, based on technology known as the scintillation proximity assay (SPA), is both high-throughput and robust. Over 250,000 compounds were tested and several leads resulting from this study, such as the anthraquinone dye Cibacron blue 3G-A, were found to sensitize MRSA to β-lactams. The compounds themselves demonstrated weak antibacterial activity. In a separate screening program, two agents (MC-200,616 (**2**) and MC-207,252) were identified which potentiate the activity of β-lactams against MRSA (31). The MIC_{90} of imipenem for 24 strains of MRSA was approximately 500-fold lower in the presence of these agents, shifting from 64 to 0.125 μg/mL. The levels of susceptibility to antibiotics of classes other than the β-lactams, such as macrolides, quinolones, glycopeptides and aminoglycosides, remained unchanged in the presence of **2**. Both compounds appear to modify the expression and/or function of PBP2a. In addition, MC-207,252 successfully potentiated the



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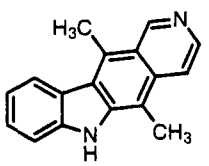
activity of a variety of β-lactams against MRSA in an *in vivo* infection model (32).

INHIBITION OF RIBOSOME-MEDIATED RESISTANCE

Macrolide-lincosamide-streptogramin (MLS) Resistance - Macrolide, lincosamide and streptogramin (MLS) antibiotics are structurally distinct but share a similar mechanism of action (33,34). They exert their antibacterial effects by binding to the 50S subunit of the bacterial ribosome, inhibiting protein synthesis by preventing transpeptidation and translocation reactions (35). The introduction of erythromycin in the 1950's was followed shortly thereafter by reports from several countries of erythromycin-resistant staphylococci (4). Studies since have established that the most common mechanism of resistance is associated with modification of the ribosome by adenine-specific N-methyltransferases (36,37). The modified ribosome binds the MLS antibiotics less efficiently leading to a co-resistance phenotype which is widely distributed and has been detected in a variety of gram-positive and gram-negative organisms such as *Staphylococcus* spp., *Streptococcus* spp. and members of the family *Enterobacteriaceae.*

The enzymes involved in the modification of the ribosome are inducibly or constitutively produced (38) and are encoded for by a class of genes called *erm* (erythromycin ribosome methylation) (36-39). Approximately 30 *erm* genes have been isolated and characterized from a variety of organisms which are clinically resistant to erythromycin, the most widely distributed of which is *ermC* (36,37,40-42). In both gram-positive and gram-negative bacteria carrying the *erm* gene, methylation of a particular adenine residue in a highly conserved region of the 23S rRNA has been implicated in MLS resistance (43). This residue is in a region of the ribosome directly involved in the formation of the peptidyl transferase center and, upon methylation, confers resistance to all known macrolides, lincosamides and streptogramin B class antibiotics.

Since MLS resistance is directly linked to the *erm* genotype, inhibition of the methyltransferase enzyme should, theoretically, potentiate the activity of the MLS antibiotics (4,15). Such an approach was recently undertaken to identify compounds that would specifically inhibit the methyltransferase encoded by *ermC* (44). The search utilized a high-throughput biochemical assay where the selectivity of the compounds for the methyl transferase was assessed by their ability to inhibit the liver enzyme catechol-*O*-methyltransferase and the prokaryotic enzyme, *EcoR I* methylase. Over 160,000 synthetic compounds were screened and several selective analogs of various structural types such as **3** and **4** were identified which had IC_{50}s ranging between 450 and 900 nM. The compounds fared poorly *in vitro*, however, in combination with azithromycin against strains of bacteria expressing MLS-resistance. Similarly, none of the compounds, in combination with azithromycin, protected mice infected with a *S. aureus* strain expressing inducible MLS-resistance at doses of 100 mg/kg.



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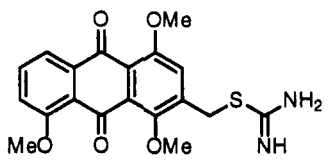

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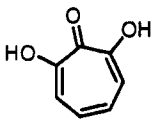
Tetracycline Resistance - Tetracyclines have been used extensively over the last 50 years to treat a variety of bacterial infections in both humans and animals. The compounds inhibit bacterial cell growth by the inhibition of protein synthesis at the level of the ribosome by disrupting the codon-anticodon interactions between tRNA and mRNA (45). Bacterial resistance to this class of antimicrobial agents is a growing problem and is primarily due to the transfer of resistance genes rather than *via* spontaneous mutations (46). The exact mechanism of resistance to tetracyclines is not completely understood, but it appears that a cytoplasmic protein associates with the ribosome and allows for protein synthesis to occur even in the presence of bound tetracycline. Like macrolides, the most prevalent mechanism of resistance to tetracyclines is ribosomal protection (14,15). The genes responsible for this are designated Tet(M), Tet(O) and Tet(Q) and typically reside in plasmids and/or transposons (45-47). Tet(M) and Tet(O) are found in a variety of gram-positive and gram-negative organisms such as *Staphylococcus* spp. while Tet(Q) is found only in *Bacteroides* (48).

INHIBITION OF ANTIBIOTIC INACTIVATION PROCESSES

Aminoglycosides - The most clinically significant contributor of bacterial resistance to aminoglycosides is plasmid-mediated enzymatic alteration of the drug (49). Three classes of aminoglycoside modifying enzymes have been identified in both gram-positive and gram-negative bacteria and include N-acetyltransferases, *O*-adenyltransferases and *O*-phosphotransferases (kinases). Over 50 aminoglycoside modifying enzymes have been characterized at the genetic level and all of the proteins studied to date are constitutively expressed (50). In contrast to resistance determinants found in macrolides, there is no evidence for inducibility of aminoglycoside resistance (51). Because the rate of modification is an important determinant of the level of resistance, it may be reasonable to expect that inhibitors of aminoglycoside modifying enzymes could be useful as potentiators of aminoglycoside antibiotics (15). However, since nearly all clinically used aminoglycosides can be modified by at least two separate mechanisms, a broad spectrum inhibitor or a combination of two inhibitors would most likely be necessary in overcoming aminoglycoside resistance.

There are examples of approaches to inhibit each of the three classes of aminoglycoside modifying enzymes described above. For instance, one study (52) examined the inhibition of gentamicin acetyltransferase-I by a gentamicin-CoA multi-substrate analog. Although the compound inhibited the acetyltransferase with a K_i = 5-20 x 10^{-10} M, it did not potentiate the activity of gentamicin against gentamicin-resistant *E. coli, in vitro.* In a separate study, 7-hydroxytropolone (**5**) potentiated the activity *in vitro* of several aminoglycosides against aminoglycoside-resistant bacteria possessing a 2″-*O*-adenyltransferase (53), and a modest enhancement of the efficacy *in vivo* of tobramycin was demonstrated (54). A variety of structurally diverse analogs of **5** were prepared; however, few of these analogs showed any significant enzymatic inhibitory activity (54).



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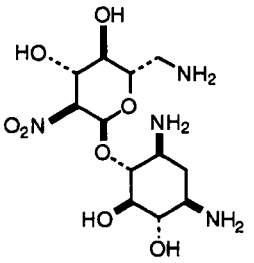
The most studied aminoglycoside-modifying enzymes are the ubiquitous aminoglycoside 3′-phosphotransferases [APH(3′)s] (50). These enzymes phosphorylate the 3′ and or 5″ hydroxyl group of a variety of aminoglycosides such as amikacin. Several compounds, such as the structurally related neamine and kanamycin analog **6**, have been described which serve as mechanism-based inhibitors

of the 3´-phosphotransferases (55). Based on a study which demonstrated that the structure of APH(3´)-IIIa is related to eukaryotic protein kinases (56), Daigle and co-workers recently evaluated known protein kinase inhibitors as potential inactivators of aminoglycoside kinases (57). Several isoquinolinesulfonamide derivatives such as **7** and **8** were found to inhibit APH(3´)-IIIa but did not reverse aminoglycoside resistance in cell culture.



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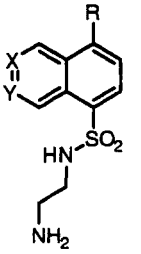


7 X= N, Y= CH, R= H
8 X= CH, Y= N, R= Cl

Chloramphenicol - The primary mechanism of resistance to chloramphenicol is due to the presence of chloramphenicol acetyltransferase (CAT), an enzyme which catalyzes the acetylCoA-dependent acetylation of the antibiotic at the C-3 hydroxyl group (58). Genes for CAT are widely distributed among most gram-negative and gram-positive bacteria and several show a high degree of homology, especially around their active sites (59). Thus, it may be possible to identify a single, broad spectrum inhibitor of all CATs which would potentiate the activity of chloramphenicol. Indeed a compound which inhibits CATs produced by *Streptomyces* and potentiates the activity of chloramphenicol *in vitro* has been identified (60). The agent, a peptide analog, is also active *in vivo*. Recently, a new class of enzymes was discovered which are structurally unrelated but functionally similar to the classical CATs (59). The enzymes, know as xenobiotic acetyltransferases (XATs), may offer new opportunities for chloramphenicol potentiation.

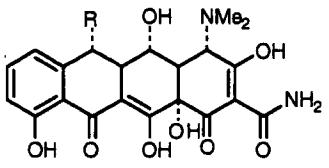
Macrolides - Various inactivating enzymes, encoded for by *ereA* and *ereB*, are responsible for high-level macrolide resistance and have been detected in strains of *E. coli* and in members of the family *Enterobacteriaceae* (61-63). The enzymes hydrolyze the lactone ring of 14-membered macrolides such as erythromycin (64). Enzymatic inactivation of macrolides is quite limited as nearly all of the macrolide resistant strains found in the clinical setting involve ribosomal modification (61).

INHIBITION OF EFFLUX PUMPS IN BACTERIA, FUNGI AND PROTOZOA

General Overview - Without doubt, drug inactivation and target modification are two of the most studied and best characterized mechanisms of resistance to antimicrobial therapy (16,17). Over the last several years, however, resistance caused by active efflux mechanisms has attracted worldwide attention since the discovery that tetracycline resistance in *E. coli* is based not just on ribosomal modification but also on energy-dependent efflux (20). Active efflux systems are recognized today as an important cause of antimicrobial resistance among a variety of structurally diverse agents, including antibacterials, antifungals and antiprotozoals (2,3,18). Thus, the inhibition of efflux transporters to potentiate the activity of antimicrobial agents is an attractive avenue of drug discovery.

Bacterial - Active efflux plays a major role in the resistance of a variety of organisms to several classes of antibacterial agents, including β-lactams, macrolides, tetracyclines and fluoroquinolones (18). Whereas tetracyclines and macrolides are excreted by the expression of drug-specific pumps (45,65-67), a variety of structurally unrelated antimicrobial agents are substrates of multidrug efflux pumps (68-70). Both drug-specific and multidrug efflux systems are found in a variety of gram-negative and gram-positive bacteria. Active efflux of antimicrobial agents has even been demonstrated in wild-type strains of enterococci and *Pseudomonas aeruginosa* (71,72).

Several approaches to potentiate the activity of antibacterial agents *via* the inhibition of efflux pumps have been described. For example, an inhibitor of efflux pumps in *Pseudomonas aeruginosa* which potentiates the activity of quinolones was recently identified using a high-throughput-screening program (73). In a separate study, assays to identify inhibitors of tetracycline efflux pumps were described which specifically identified tetracyclines of distinct structure (74). Also, various structural analogs of tetracycline were tested in an everted membrane vesicle system for their ability to inhibit the tetracycline efflux pump (75,76). One compound, 13-[(3-chloropropyl)thio]-5-hydroxy-tetracycline (**9**), demonstrated the best efflux inhibitory activity and exhibited potentiation *in vitro* against various organisms which are resistant to tetracycline due to an efflux mechanism. This study was unique in that it revealed both extensive and important SAR information regarding a specific class of efflux pump inhibitors.



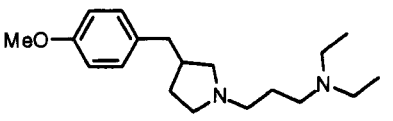
9 R = $CH_2S(CH_2)_3Cl$

Fungal - The azole antifungal agents, such as ketoconazole, fluconzaole and itraconazole, inhibit the cytochrome P450-dependent 14α-demethylase, an essential enzyme of the ergosterol biosynthetic pathway (77). As a consequence of the increasing number of infections caused by *Candida albicans*, especially among AIDS patients, the use of azole antifungals has increased and has led to the appearance of yeast isolates resistant to these agents (78,79). There is growing evidence that efflux of the azole antifungal agents is an important mechanism of resistance in various isolates of *Candida* (80-83). To date, three multidrug efflux transporters, the ATP-binding cassette (ABC) transporters, *CDR1* and *CDR2*, and the major facilitator *BEN* have been identified (84-86). Overexpression of the proteins encoded for by these genes results in an increased efflux of various azoles from a variety of fungal species (87-88). Interestingly, the genes encoding members of the superfamily ABC transporters are well known mediators of multidrug resistance (MDR) in mammalian cells (89). The mechanism of MDR has been extensively studied and involves the expression of p-glycoprotein (P-gp), an efflux pump for various antineoplastic agents (90). The potentiation of antitumor agents by inhibition of P-gp using a variety of agents such as verapamil and MS-209 has been reported to reverse MDR both *in vitro* and *in vivo* (90-92).

Protozoal - The emergence of resistance in the late 1950's to chloroquine, an agent used to treat malaria, severely compromised the effectiveness and use of this drug (3). Studies have demonstrated that chloroquine resistance in *Plasmodium falciparum*, the causative organism, bears close similarities to the MDR phenotype described above and can be reversed by several drugs including verapamil (3). Two genes, *pfmdr1* and *pfmdr2*, have been identified in *P. falciparum* which are

approximately 60% homologous to the MDR genes found in mammalian cells (93,94). However, the exact role of these genes in the emergence of drug resistance remains controversial since there appears to be no correlation between the amplification of the *pfmdr1* gene and resistance to chloroquine, *in vitro* (95).

With growing evidence that chloroquine resistance patterns are modulated *via* a P-gp-like transporter, studies to block the MDR phenotype and potentiate the activity of chloroquine have been reported. For example, chlorpheniramine reverses chloroquine resistance in 11 of 14 *P. falciparum* isolates at 625 nM with no potentiation observed against chloroquine-susceptible clones (96). In another study, fangchinoline, a bis-biphenylisoquinoline, potentiated the activity of chloroquine against a chloroquine-resistant *P. falciparum* strain *in vitro* (97). The compound also potentiated the activity of vinblastine in an MDR cell line approximately 90-fold, indicating it may inhibit the P-gp transporter. WR268954 (**10**), a pyrrolidino alkylamine, decreases the IC_{50} of chloroquine for drug resistant *P. falciparum* 90-fold when compared to chloroquine alone (98). The compound has weak intrinsic antimalarial activity and may act as a competitive inhibitor of the binding of chloroquine to the putative transporter.



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VANCOMYCIN RESISTANCE IN ENTEROCOCCI

Resistance to vancomycin does not fit within any of the categories described above, due to the unique mechanisms of action of the glycopeptide class of antibiotics (99). Vancomycin inhibits peptidoglycan synthesis by binding to the D-Ala-D-Ala terminus of the disaccharyl pentapeptide unit of the peptidoglycan polymer comprising the bacterial cell wall. Over the last several years, clinically significant vancomycin resistance in enterococci has appeared and has been traced to a set of five genes, VanA, VanH, VanR, VanS and VanX (99,100). Each of the genes is required, and together are sufficient to confer the resistance phenotype. Whereas VanH and VanA synthesize D-Ala-D-lactate, which is incorporated into the peptidoglycan chain in place of D-Ala-D-Ala, VanX hydrolyses D-Ala-D-Ala and dramatically reduces the availability of precursors ending in D-Ala. Synthesis of VanA, VanH and VanX is regulated at the transcriptional level by the VanR-VanS two-component regulatory system.

Theoretically, inhibition of any or all of the five important Van genes would inhibit the resistance mechanism and potentiate the activity of vancomycin. Several reports describing such approaches are known. For example, several phosphinates inhibited the enzymatic activity of VanX by acting as slow binding inhibitors (101,102). One compound, D-3-[(1-aminoethyl)phosphinyl)]-D-2-methylpropionic acid, showed a time-dependent onset of inhibition of VanX but failed to potentiate the activity of vancomycin in a strain of vancomycin-resistant *Enterococcus faecium*. In a similar study, several compounds such as 2,3-dimercapto-1-propanesulfonic acid and 2,3-dimercapto-1-propanol inhibited VanX in a time-dependent fashion (103). In a separate study, the MIC of vancomycin in several resistant isolates was reduced up to 31-fold in the presence of an amphipathic cationic peptide (104). The compound exhibited little to no intrinsic antibacterial activity at concentrations equal to or less than 32 µg/mL.

CONCLUSION

With an ever increasing understanding of the mechanisms of antimicrobial resistance, the discovery and development of potentiators of known antimicrobial agents should be included alongside the elucidation of structurally unique agents and the identification of novel targets. The success of β-lactamase inhibitor/β-lactam combinations provides compelling evidence for the feasibility of just such an approach.

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Chapter 13. Matrix Metalloproteinase Inhibitors and Cancer

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Introduction – Cancer claims the lives of more than six million people worldwide each year; one in five Americans will experience cancer in their lifetime and one in three will die from it. Despite billions of dollars in research over several decades, little progress has been made to reduce these macabre statistics. Often ineffective cytotoxic drugs, radiation and surgery together with their frequently associated sequelae of debilitating side effects are still the mainstays of cancer therapy. However, there is reason for hope. Several promising approaches have appeared on the horizon in recent years which offer the potential for a more effective and less toxic treatment for cancer. Among these are the inhibitors of the matrix metalloproteinases (MMP). Evidence summarized herein suggests that MMP inhibitors may prevent local invasion and distant metastasis of cancer cells as well as limiting tumor growth by blocking new blood vessel formation and the activation of growth factors. A conclusive link between MMPs and cancer may soon emerge as the results of definitive clinical trials with inhibitors are completed.

The MMPs have been extensively reviewed (1-4) including a recent general Report within this series (5). Although MMPs may play a role in numerous diseases including arthritis, periodontal disease, multiple sclerosis and osteoporosis, this Report will focus exclusively on the recent developments relating MMPs to cancer.

Overview of the MMPs – The matrix metalloproteinases are a family of zinc containing enzymes that degrade extracellular matrix. They are excreted by a variety of connective tissue and pro-inflammatory cells including fibroblasts, osteoblasts endothelial cells, macrophages, neutrophils and lymphocytes. Most are excreted as inactive proenzymes and then activated extracellularly by serine proteases or other MMPs. These activated enzymes are inhibited by secreted proteins known as tissue inhibitors of metalloproteinases (TIMP) of which there are currently four known (6). The relative expression of TIMPs and MMPs in tissues is critical to the regulation of extracellular matrix degradation and remodeling. A disruption of the inhibitor/enzyme balance contributes directly to cancer progression and other diseases.

The MMPs have been divided into four families by virtue of similarities of their domain structure (3). The smallest MMP, matrilysin (PUMP-1, MMP-7), is the only member of the Minimal Domain family and consists of a signal sequence, a prodomain with a "cysteine switch" that is removed during activation, and a zinc containing catalytic domain. This enzyme cleaves several substrates including proteoglycan, laminin, and fibronectin. The addition of a hemopexin binding domain connected by a hinge region to the C-terminus of the catalytic domain characterizes the Hemopexin Domain family. Three collagenases, so named because of their unique ability to cleave fibrillar collagen are members of this family: fibroblast collagenase (interstitial collagenase, MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). Metalloelastase (MMP-12) and stromelysin-1, 2 and 3 (MMP-3, 10, 11) also contain

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hemopexin domains. The latter member, stromelysin-3, contains a putative furin-like enzyme recognition sequence that may contribute to its secretion from cells in a catalytically active form. The proteolytic function of stromelysin-3 is unclear, but it may activate other MMPs. As the name implies, the Fibronectin Domain family of MMPs, including gelatinase A and B (72kD gelatinase, MMP-2 and 92kD gelatinase, MMP-9) contain fibronectin-like sequences within the catalytic domain and are capable of degrading a broad collection of matrix substrates including gelatin, type IV collagen and elastin. The most recently discovered class of MMPs are Transmembrane Domain family. There are currently four family members (MT-1-MMP – MT-4-MMP, MMP-14 – MMP-17). These enzymes contain a sequence that is capable of spanning the cellular membrane and are proposed to activate gelatinase A at the surface of cells (7).

New MMPs are continuing to be discovered. Using a MMP similarity search of the EST database, a partial cDNA clone that encodes the 3-prime end of a putative MMP, designated MMP-18, was identified (8). It is appears to be another Hemopexin Domain family member. Recently reported MMP-19 (9) and MMP-20 (enalmelysin) (10) also share structural elements with this family.

RATIONALE FOR MMPS IN CANCER

The role of the MMPs in the progression of cancer may involve several mechanisms. Originally the MMPs were thought to mediate invasion and metastasis primarily by matrix remodelling thereby allowing tumor cells access to blood and lymphatic vessels. Evidence for this mechanism is based largely on the increased invasiveness of cell lines which overexpress the MMPs (11,12). More recently it has been shown that MMPs can play a role in primary tumor growth (13). This may involve the release of stroma-bound growth factors or MMP-mediated tumor angiogenesis. Alternatively, remodelling of the extracellular matrix in the vicinity of the primary tumor may provide the spacial requirements necessary for tumor growth.

Much of the experimental evidence which links the MMPs with the progression of cancer comes from studies which have demonstrated elevated levels of the MMPs in human cancer biopsies. Virtually all the MMPs have been detected either at the protein or nucleic acid level in cancerous tissue, though the expression of a given MMP is not necessarily restricted to a specific tumor type. For example increased gelatinase-A expression has been demonstrated in carcinomas of the pancreas (14), breast (15) ovary (16) and colon (17) and fibroblast collagenase mRNA levels are higher in head and neck cancerous tissue relative to the corresponding noncancerous tissue (18, 19). The detection of MMP mRNA directly in cancer specimens by *in situ* hybridization indicates that most MMPs are produced by stromal rather than neoplastic cells (20). This implicates the release of cancer cell-derived factors as stimulators of stromal MMP activity and emphasizes the importance of tumor-stromal interactions in the progression of solid tumors (21,22).

Elevated MMP activity in human cancerous tissues has been correlated with tumor behavior. For example, enhanced gelatinase activity is associated with a higher tumor grade in breast (15), bladder (23) and gastric cancers (24). In colorectal cancer, tumor expression of fibroblast collagenase has been correlated with poor prognosis (25) and stromelysin-3 mRNA levels correlate with decreased patient survival in breast cancer (26). Measurement of plasma MMP concentrations, either alone or complexed with TIMP, has been examined as a noninvasive method for cancer diagnosis and monitoring (27).

While these studies provide useful correlative evidence in support of the role of MMPs in cancer, it is important to emphasize that a complete analysis of *all* MMPs expressed by a broad range of tumor types is currently not available. This raises the question as to which MMPs are primarily involved in the progression of cancer and whether these MMPs differ from those responsible for physiological processes such as tissue remodelling. Despite these uncertainties, it is clear that some MMPs are associated with human cancers more often than others. The gelatinases fall into this category and may be particularly important in tumor progression for several reasons. First, type IV collagen is a major component of basement membranes and is a particularly good substrate for both gelatinases. It has also been shown that activation of pro-gelatinase A is distinct from most pro-MMPs and involves the formation of a ternary complex between gelatinase A, TIMP-2 and MT-MMP-1 at the cell surface (28, 29). Since pro-gelatinase A is mainly expressed by fibroblasts, this suggests a mechanism by which tumors can use host derived MMPs to facilitate invasion. Consistent with this mechanism are recent results demonstrating the binding of gelatinase A to the surface of invasive cells via interaction with the adhesion molecule $\alpha v\beta 3$ (30).

MMP INHIBITORS IN PRECLINICAL STUDIES

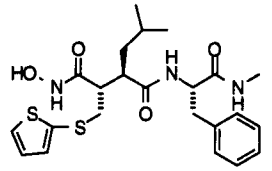
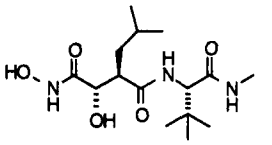
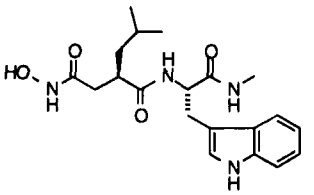
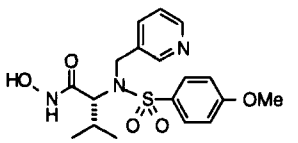
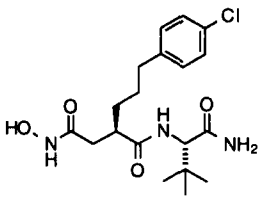
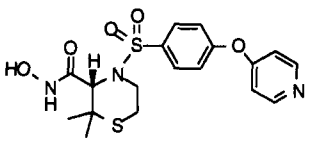
Perhaps the most convincing evidence that the MMPs play a significant role in the progression of cancer comes from studies with various MMP inhibitors. Both protein-based and small molecule synthetic MMP inhibitors have been analyzed in a variety of *in vitro* and *in vivo* models of connective tissue remodeling. The discussion of MMP inhibitors provided below will focus primarily on the activity of these agents in short term models of cancer progression.

Protein-based MMP Inhibitors – As mentioned previously, the balance between activated MMPs and TIMPs determines the overall MMP proteolytic activity and consequently, the extent of extracellular matrix turnover. Alteration of this balance in favor of excess inhibitor has been shown to modify invasive behavior. For example, exogenously added TIMP-2 has been shown to block the invasion of human fibrosarcoma HT-1080 tumor cells through a reconstituted basement membrane (31). The same is true for monoclonal antibodies raised to either gelatinase A (32) or gelatinase B (33). Intraperitoneal administration of TIMP-1 in nude mice significantly reduced lung colonization by murine melanoma cells in a model of metastasis (34, 35). Furthermore, over-expression of the TIMPs in tumorigenic cell lines markedly reduced metastatic potential and invasive behavior (36-38). TIMP-1 has been suggested to have a suppressive effect on the process of angiogenesis since it has been shown to block endothelial cell migration in a Boyden chamber assay (39).

Synthetic MMP Inhibitors – Several excellent reviews have been published which describe the various classes of synthetic MMP inhibitors as well as the structural features necessary for potent MMP inhibition (40-42). For that reason the current discussion will focus on compounds which have been examined in preclinical cancer animal models or which are currently undergoing cancer clinical trials. Table 1 provides a list of these agents along with inhibition constants for four representative MMPs. These compounds are all prime-side, reversible MMP inhibitors which rely on a hydroxamate moiety to bind to the zinc atom at the enzyme active site. Inhibition potencies vary substantially for these compounds, not only for a given enzyme but also across the MMPs listed. While batimastat (BB-94, **1**) and CGS 27023A (**4**) exhibit broad spectrum MMP inhibition, CT-1746 (**5**) and AG-3340 (**6**) are more selective for the

inhibition of the gelatinases. This raises one of the key issues in the field, namely whether broad spectrum or sub-type selective MMP inhibitors provide the optimal inhibition profile for anticancer activity. In support of the broad spectrum approach is the overlap in substrate activity between the different MMPs. Selective inhibitors may minimize potential side effects though no single MMP has been associated with the progression of a particular tumor type. It seems likely that the MMP selectivity issue will be resolved only after clinical studies of agents with differing inhibition profiles.

Table 1. Enzymatic Potency of Selected MMP Inhibitors (IC_{50}, nM)

MMP Inhibitor		MMP-1	MMP-2	MMP-3	MMP-9	ref.
	**1**	3	4	20	4	(43)
	**2**	5	6	200	3	(40)
	**3**	0.4	0.5	27	0.2	(44)
	**4**	33	20	43	8	(45)
	**5**	122	0.04	10.9	0.42	(46)
	**6**	8.2	0.083	0.17	-	(47)

Another selectivity issue relates to the activity of these compounds against enzymes which are structurally related to the matrix metalloproteinases. Succinyl hydroxamate-based inhibitors such as batimastat exhibit minimal inhibition of metalloproteinases like angiotensin converting enzyme (48) and enkephalinase (49), although related compounds have been shown to inhibit the processing of several membrane-bound ligands (50). Of particular interest is the ability of hydroxamic acid-

based inhibitors (51-53) to block the release of TNFα through inhibition of TNFα converting enzyme (TACE), a member of the reprolysin subfamily of zinc metalloproteinases (54, 55). The ramifications of this additional inhibitory activity on the anticancer properties of these inhibitors is currently unknown.

By far the most well-studied inhibitor in terms of preclinical cancer animal models is batimastat. In a syngeneic model of experimental metastasis, batimastat was shown to inhibit the number of lung colonies produced by intravenous injection of B16-BL6 mouse melanoma cells (56). The growth of B16-BL6-derived tumors following subcutaneous inoculation was inhibited even though batimastat demonstrated no direct cytotoxic effects *in vitro*. Efficacy has also been demonstrated for batimastat in xenografts models of human cancers. The compound given by intraperitoneal injection at 50 mg/kg/day caused a reduction in the volume of human breast cancer solid tumors grown in the mammary fat pad of nude mice (57). Human MDA-MB-435 breast cancer cells were used to demonstrate the inhibitory activity of batimastat on lung metastasis following resection of the primary tumor (58). An increase in survival time has been demonstrated with batimastat in xenograft models of human ovarian (59) and pancreatic cancer (60).

The antiangiogenic properties of batimastat have been investigated using several models (61). Endothelial cell invasion through a layer of reconstituted basement membranes (Matrigel) was significantly inhibited in the presence of batimastat despite its minimal effect on cell proliferation. Transformed mouse endothelial cells were used to form hemangiomas in nude mice and batimastat dose-dependently inhibited the growth of these tumors while reducing tumor associated hemorrhage. These results suggest that the growth inhibitory properties of MMP inhibitors may be at least partially mediated by alterations of the angiogenic response.

GM-6001 (**3**) is another broad spectrum MMP inhibitor which exhibits antiangiogenic activity, in this case as measured by a reduction in blood vessel number in Hydron pellets implanted in the corneas of rats (62). Treatment of a c-Ha-ras transfected glial cell line with **3** reduced the percent of cells migrating through reconstituted basement membrane and implicates the MMPs in brain tumor invasion (63). Like batimastat, **3** exhibits poor bioavailability in primates and consequently its clinical evaluation has been limited to diseases which are consistent with topical administration, for instance corneal ulceration (64).

Not all hydroxamate-based MMP inhibitors have limited bioavailability. Marimastat (BB-2516, **2**) is 20 to 50% orally available in laboratory animals depending on the species (48) though mini-pumps are required to sustain therapeutic blood concentrations in rodents (1). It has been speculated that internal hydrogen bonding of the α-hydroxy substituent and a shielding effect of the *tert*-butyl group contribute to the enhanced oral absorption of marimastat (40). A similar explanation has been proposed for the superior pharmacokinetics displayed by the D-valine-derived sulfonamide CGS 27023A (**4**) relative to its structural analogs. That is, the large isobutyl group protects the hydroxamate moiety from metabolic degradation (45). While both of these broad spectrum MMP inhibitors are currently undergoing cancer clinical studies, published data in preclinical cancer animal models have not appeared.

Gelatinase selective MMP inhibitors which possess pharmacokinetics sufficient for *in vivo* efficacy studies have been difficult to identify, yet at least two have been reported in the literature. CT1746 (**5**) dosed in combination with the

cytotoxic agent cyclophosphamide was more effective at inhibiting the growth and metastasis of Lewis lung tumors than either agent used alone (65). In a model of human colon cancer involving the orthotopic implantation of histologically-intact human tumor tissue, **5** prolonged the median survival time in addition to inhibiting primary tumor growth and metastatic spread (66). The relationship between rodent pharmacokinetics and anti-tumor effects of a series of gelatinase selective MMP inhibitors have been studied using the murine Lewis lung carcinoma model (67). While a definitive correlation between these two variables could not be constructed, AG3340 (**6**) was identified as the most efficacious analog within the series. At a dose of 50 mg/kg once daily, **6** caused complete cessation of primary tumor growth in 66% of treated mice. Clinical development of this compound is currently underway.

CLINICAL TRIALS WITH MMP INHIBITORS

Although several MMP inhibitors are now engaged in clinical trials for the treatment of cancer, only the results of trials with batimastat and marimastat have been reported in detail.

Batimastat – Because of its low aqueous solubility, batimastat could not readily be administered orally or intravenously to humans. Therefore, initial clinical studies were focused on cancers where the drug could be delivered parenterally in the vicinity of a relevant tumor. Batimistat was administered as a suspension through a cannula directly into the peritoneal or pleural cavity in patients with malignant ascites or pleural effusions. Intraperitoneal administration gave rise to high and sustained plasma concentrations that peaked within 24–48 hours and were still detectable for up to four weeks after dosing (68). An effective half-life of 19 days was estimated (69).

Although it is not possible to assess efficacy in any of these studies, a suggestion of clinical benefit was observed. When batimastat was administered intraplurally, a significant improvement in dyspnea and reduction in the number of pleural aspirations in one month were noted (70). Following intraperitoneal delivery, five of nine patients with ascites responded to the drug as assessed by a reduction in abdominal girth and drainage (71).

The observation that intraperitoneal administration of batimastat created a depot that was slowly dissolved to produce sustained systemic exposure, lead to the evaluation of the drug in patients with other types of malignancies (72). Although initial trials suggested no major toxicities in patients with malignant ascites, intraperitoneal injection in individuals without ascites resulted in substantial abdominal pain and cramping that was attributed to deposition of the insoluble drug in the peritoneum. Acute bowel toxicity was seen in significant proportion of patients in a larger study (69). In view of these toxicities and because batimastat could only be administered directly to a body cavity, it was suspended from clinical trials for cancer in late 1996 in favor of the orally active analog marimastat (73).

Marimastat – Initial clinical trials confirmed the oral bioavailability of marimastat in healthy volunteers that was predicted from animal studies (74). The drug was well absorbed, with an estimated bioavailability of approximately 70%. The elimination half-life was estimated to be between 8–12 hours, consistent with twice daily dosing. Plasma levels were approximately 2- to 3-fold higher in cancer patients than in healthy individuals, perhaps because of the age of the subjects and decreased clearance

rates (69). Following 10 mg, b.i.d., trough plasma concentrations of marimastat remained more than 40 times the IC_{50} of the compound versus gelatinase A.

The evaluation of the efficacy of an MMP inhibitor in cancer is more challenging than that of traditional cytotoxic agents. Since MMP inhibitors are expected to act through prevention or reduction of further growth, not by killing the tumor, examination of tumor reduction is not appropriate. Ultimately these type of drugs must demonstrate increased survival or decreased time to disease progression, but these studies are large, expensive and time consuming. Therefore, the first efficacy studies with marimastat were designed to utilize biological markers which might provide an indication of therapeutic potential and optimal dose. Since an increase in the level of plasma cancer specific antigens (e.g., PSA for prostate cancer) may reflect a progression of the tumor, phase II clinical trials with marimastat were designed to evaluate the rate in rise of these markers. In a series of trials involving various types of cancers, patients were selected who exhibited at least a 25% increase in the rate of rise of plasma cancer antigen over the month prior to initiation of the therapy (75-78). Marimastat treatment significantly reduced the rate of rise of all antigens examined in a dose dependent manner. More than 50% of the patients exhibited a diminished or reduced rise in antigens. The rate of antigen level rise rebounded seven days after the drug was discontinued. In addition, these changes were correlated to a decrease in tumor burden by CT scan in pancreatic cancer (75, 79) and to an increase in survival in ovarian (78) and pancreatic patients. The design and the relevance of these trials has been debated in the literature (80). It was argued that the fall in the rate in rise of cancer antigens may merely reflect the normal marked variation in levels observed over a short interval. This debate will only begin to be resolved when larger clinical trials are completed that examine the influence of MMP inhibitors on established endpoints such as survival in comparison to standard therapies. Five phase III trials with marimastat in a variety of cancers are currently in progress.

Marimastat is generally well tolerated with the exception of a musculoskeletal syndrome characterized by pain and stiffness usually commencing in the small joints of the hand and spreading to the arms and the shoulder (43, 69). If dosing continues other joints may become involved as well, but the symptoms disappear within 1–3 weeks after the drug is discontinued. The side effects are dose and time dependent; at the lowest dose that significantly reduced cancer antigens (10 mg, b.i.d.) approximately 30% of the patients develop it after 3–5 months of treatment. To manage this toxicity, subsequent clinical trials are incorporating a short "dosing holiday" followed by a dose reduction. The symptomatology of the musculoskeletal effect is similar to that seen in toxicological studies in the marmoset that involved tendinitis at the tendonis insertion to the joint. The cause of this toxicity is unknown, but may indicate an impairment of the normal tissue remodeling that occurs regularly in the tendons and joints. It has been speculated that this remodeling may be governed by one or more of the MMPs. Although the precise identity of which MMPs, if any, are responsible for the toxicity is unclear, several laboratories have focused on the development of inhibitors that unlike marimastat selectively block only a subset of MMPs.

Other Inhibitors – Several other MMP inhibitors are involved in cancer trials including **4** and **6**. Phase I results with the latter compound have been reported (81). The drug was well absorbed and displayed a half life of between 2.8 and 5.9 hour following a single dose in healthy volunteers.

CONCLUSIONS

The preclinical data available to date, strongly support a role for MMPs in several aspects of cancer. Based on work cited here and elsewhere, there is reason to believe that MMP inhibitors may influence the local invasion and metastatic spread of tumors as well as control growth by blocking new blood vessel formation. However, a definitive link of MMPs to cancer cannot be established until it has been demonstrated in large, well designed clinical trials that inhibitors have a significant effect on patient survival or time to disease progression. Hopefully, this puzzle piece should be put in place in the near future as results of such trials become available.

Although the first generation of compounds may establish a definitive link between cancer and MMPs, there will still be much that remains to be answered. The musculoskeletal side effects seen in early trials may prove to be an impediment to long term dosing, compliance and optimal efficacy. As such it may be critical to identify agents which control tumors without significant side effects. Doing so will require a more precise understanding of the relationship of MMPs, tumors and tendon remodeling. It remains to be seen whether selective inhibition of a subset of MMPs will provide adequate efficacy in a broad range of cancer, avoid side effects, and evade the development of resistant mechanisms.

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Chapter 14. Recent Developments in Antibacterial Research

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Introduction - Bacterial resistance to a number of antimicrobial agents such as vancomycin, quinolones, macrolides and ß-lactams is becoming a serious worldwide health problem (1-4). The major problems in clinical practice involving bacterial infections are the increase in the isolation of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and penicillin-resistant *Streptococcus pneumoniae.* Multidrug-resistant strains of *Mycobacterium tuberculosis* have emerged in several countries including the United States (5). This rapid and extensive emergence of antibiotic resistant bacteria has resulted in a clear cut need to discover new antibiotics. To accomplish this, scientific and medical communities have engaged in research to search for improved agents within known antibiotic classes and novel agents without cross-resistance by having novel bacterial targets. Studies on bacterial genomics for target identification and the biochemical basis of bacterial resistance mechanisms for a rational approach to the solution of the resistance problem are being actively pursued. Reviews on different approaches to new antibacterial agents to overcome bacterial resistance have been published (6-8).

BACTERIAL RESISTANCE

Interactions of bacterial resistance gene transfer elements and their stability may explain the difficulty to achieve resistance reversal and the need to have epidemiological studies on both clinical and non-clinical isolates (9). Reviews on carbapenem-hydrolyzing ß-lactamases (10) and the regulation of chromosomally mediated multiple antibiotic resistance [the *mar* regulon (11)] have appeared. Biology and molecular actions of the quinolone target enzymes DNA gyrase and bacterial topoisomerase IV were reviewed (12-14). Reviews on modes of action of various antibacterial agents (15), streptogramins (16,17) and membrane-acting antimicrobial peptides (18,19) were published.

Quinolone - Apart from quinolone resistance resulting from mutations in the structural genes (*gyrA*, *gyrB*, and *grlA*), a novel *S. aureus recG* gene was found to affect the quinolone susceptibility in *S. aureus*. It was cloned and sequenced and thought to be involved in the repair of DNA damage resulting from quinolone treatment (20). The importance of NorA in the susceptibility of *S. aureus* to quinolones was again demonstrated by a study on a *norA*- disrupted *S. aureus* (21,22). Studies on the inhibitory activities of quinolones against DNA gyrase and topoisomerase IV purified from *S. aureus* reconfirmed the notion that mutations in *grlA* and subsequently in *gyrA* confer high-level resistance in *S. aureus* (23,24). High-level fluoroquinolone resistance in *Streptococcus pneumoniae* was also found to require mutations in *parC* and *gyrA* (25). Low-level resistance was associated with *parC* mutations [Ser79Tyr, Ser79Phe or Asp83Gly](26). Mutation in *parE* genes of two mutants of *S. pneumoniae* was also found to be responsible for low-level resistance to fluoroquinolones. Sequential acquisition of mutations in *parE* and *gyrA* leads to higher level of resistance confirming that topoisomerase IV is the primary target of fluoroquinolones in *S. pneumoniae* (27). In contrast to the results obtained by studies on ciprofloxacin resistance in the Gram-positive pathogens *S. aureus* and *S. pneumoniae*, the primary target for sparfloxacin in *S. pneumoniae* is DNA gyrase and not topoisomerasese IV (28), very much like the usual quinolone resistance in *E. coli* (29). Active efflux as an alternate mechanism of resistance to quinolone in *S. pneumoniae* was confirmed (30). While multidrug efflux system is mainly used by quinolone resistant *Pseudomonas aeruginosa* (31), high-level quinolone-resistant isolates had a double amino acid change in GyrA and a single

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amino acid change in ParC (32). A recent study reported that a multidrug transporter may also be involved in the quinolone resistance of *S. pneumoniae* (33). The *gyrA* gene of *Campylobacter fetus* was cloned and sequenced with substitution of Asp-91 to Tyr in gyrA in a ciprofloxacin resistant strain (34). A mutation at position 83 from Ser to Arg or at position 87 from Glu to Gly was found in *E. faecalis* clinical isolates (35). Alterations in the GyrA and ParC were also found in quinolone-resistant clinical isolates of *Klebsiella pneumoniae* (36,37). A double mutation in *gyrA* in a mutant with high-level resistance was isolated (38). Resistance to quinolone occurring in environmental population bacteria was in part due to natural sequence variation in a quinolone resistance-determining region of gyrA gene (39). Adaptive mutations producing resistance to ciprofloxacin was identified (40).

Glycopeptide - While the mechanism of glycopeptide resistance in *S. aureus* is unknown, increased production of penicillin-binding protein 2 (PBP2) and other PBPs was observed in laboratory induced glycopeptide-resistant *S. aureus* mutants (41). Identification and characterization of an insertion sequence-like element, designated IS*1476*, in a VanA *Enterococcus faecium* clinical isolate was reported. It disrupts VanY function reducing the VanY D,D,-carboxypeptidase activity (42). A study of geographic differences on the source of VRE, in contrast to findings in Europe, failed to find evidence of VanA- or VanB-type VRE in the community or environmental sources in Houston, Texas (43). A new type of glycopeptide resistant phenotype, designated VanD for the constitutively resistance in *E. faecium* BM4339 was reported (44). Transfer of *vanB* among enterococci can substantially alter the expression of glycopeptide resistance by transconjugants (45). A report on the emergence of vancomycin resistance in *Streptococcus bovis* having a *vanB* transferable determinant revealed for the first time the role of streptococci in the dissemination of vancomycin resistance among Gram-positive bacteria (46).

Macrolide - High incidence (24-82%) of erythromycin-resistant streptococci was reported in Taiwan (47). PCR technique was used to distinguish the different mechanisms (efflux pumps, Erm methylases and inactivating enzymes) of erythromycin resistance (48). A significant number of erythromycin-resistant *S. pneumoniae* and *S. pyogenes* strains contain a determinant that mediates resistance via a putative efflux pump. The *mefE* gene (90% sequence identity to *mefA* in *S. pyogenes*) was found to be present in erythromycin-resistant M phenotype (macrolide resistant but clindamycin and streptogramin B susceptible) in *S. pneumoniae* (49). A novel macrolide efflux gene, *mreA*, distinct from the *mef* in *S. pneumoniae* and *S. pyogenes* and the multicomponent *mrsA* in *S. aureus* was found in a strain of S. *agalactiae* which displayed resistance to 14-, 15- and 16- membered macrolides (50). Mutations at position 2143 (A-to-G or A-to-C) of the *H. pylori* 23S rRNA confer high-level resistance while mutation at position 2144 (A-to-G) produced a stain with MIC≤ 32 μg/ml (51, 53). This point mutation confers resistance to macrolides by disruption of the peptidyltransferase loop conformation, reducing drug binding to the ribosomes (54). Similar to macrolide resistance in *H. pylori*, high-level resistance to erythromycin in cutaneous propioibacteria was also determined to have mutations (A-to-G) at either position congate with *E. coli* 23S rRNA base 2058 and 2059. Low-level resistance with G-to-A transition at base 2057 was also found (55). A study on *erm* inducers found that *ermSV* in *Streptomyces viridochromogenes*, unlike *ermC* in *S. aureus*, can be induced by the 16-membered-ring macrolide tylosin; the major factor which determines whether a given macrolide induces resistance is its size (56). The effect of topical use of erythromycin on ecology of aerobic cutaneous bacteria flora was found to be transient and of no serious consequence (57).

ß-Lactam - Resistance to extended-spectrum cephalosporins in *enterobacteriaceae* is increasingly associated with extended spectrum plasmid or transposon-mediated ß-lactamases, being mutants of molecular class A ß-lactamases (TEM-1, TEM-2 and SHV-1). Other extended spectrum ß-lactamases include the class D ß-lactamase (OXA). Several new ß-lactamases and ß-lactamase encoding genes were isolated

and characterized (58-70). Constitutive ß-lactamase production in *E. faecalis* is due not only to the absence of regulatory *blaR1* and *bla1* genes but some other additional factors (71). A study on the enhanced ß-lactamase expression in *P. aeruginosa* isolates from patients with cystic fibrosis shows that it is due to mutation in the regulatory proteins other than AmpR (72). A susceptibility study on *P. aeruginosa* strain with overproducing PBP3 confirmed the earlier reports that *P. aeruginosa* PBP3 was the primary target for extended-spectrum cephalosporins (73). A mutation in the D, D-carboxypeptidase PBP3 of *S. pneumon-iae* was found to contribute to cefotaxime resistance (74). Outer membrane porin alteration is involved in ß-lactam resistance in *Enterobacter aerogenes* (75), *Shigella dysenteriae* (76), *citrobacter freundii* (77) and *Klebsiella pneumoniae* (78). Wide-spread occurence of PER-1 extended-spectrum ß-lactamases among nosocomial *Acinetobacter* and *P. aeruginosa* in Turkey (79), Toho-1-like ß-lactamases in *E. coli* in Japan (80) and ß-lactamase-mediated ampicillin resistance in *H. influenzae* in US are seen (81).

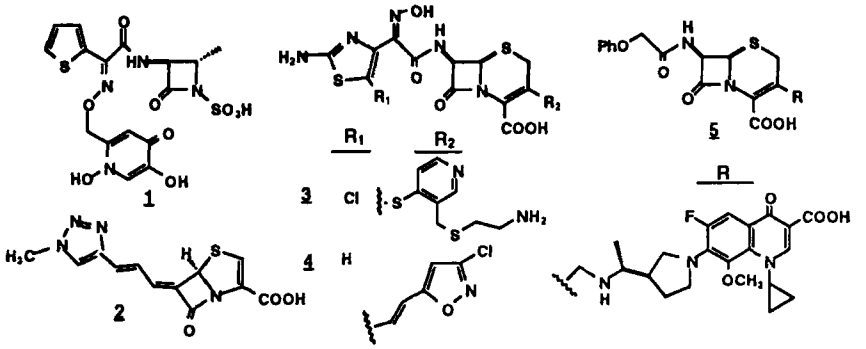
Antimycobacterial Agents - The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* complex and the resistance to isoniazid (INH) is an increasing problem for tuberculosis control programs. Point mutations at codon 315 of *katG* gene, encoding catalase-peroxidase were found in genomes of 64% of INH-resistant strains in Africa (82). Although over-expression of alkyl hydroperoxide reductase (AhpC) increases the MIC of INH, INH resistant strains with mutation of *katG* lack alterations in the *ahpC* gene, or *oxyR-ahpC* intervening region (83, 84). Mutations in *emrB* gene, which encodes the putative targets of antimycobacterial agent ethambutol arabinosyl transferases, is responsible for resistance to ethambutol in mycobacteria and *Mycobacterium smegmatis* (85, 86). The mutations in *pncA* in pyrazinamide-resistant *M. tuberculosis* were characterized (87,88). Although a report on a class A ß-lactamase expression correlated with the increased resistance phenotype of *M. tuberculosis* H37Ra (89), study on a mutant of *M. smegmatis* did not observe the increase in ß-lactamase activity. Decreased affinities of PBP 1 for ß-lactams, combined with the decreased permeability of the cell wall was proposed as the resistant mechanism (90). Multations in *rpsL* which encodes ribosomal protein S12, were associated with high-level resistance in Streptomycin-resistant *M. tuberculosis* (91).

Miscellaneous - Aminoglycoside 6'-N-acetyltransferase genes have been cloned and characterized from *C. freundii* (92, 93) and from *E. faecium* (94). A novel gentamicin resistance gene *aph(2")-Ic* which encodes phosphotransferase Aph(2")-Ic was identified in enterococci (95). Pheromone-responding plasmids encoding gentamicin resistance were isolated from multiresistant *E. faecalis* isolates in Japan (96). Chromosomal location for the *murA* gene conferring low-level mupirocin resistance to mupirocin was identified the first time from *S. aureus* (97). A conjugative plasmid encoded *mupA* gene, pXU10, encoding high-level mupirocin resistance was transferred from *S. haemolyticus* isolate to other coagulase-negative staphylococci (98). The tetracycline resistance gene *tet* (O) was detected for the first time in the *S. pneumoniae* and found to share 99% nucleotide sequence identity with the *tet* (O) resistance genes of *Streptococcus mutans*, *Campylobacter coli* and *Campylobacter jejuni* (99). The resistant dihydrofolate reductase genes from *S. pneumoniae* isolates were found to be highly conserved redundant changes in nucleotide sequence (100), and the multidrug resistance efflux gene *oprM* in *Pseudomonas* was found to be very conserved (101). Active efflux of quinolones and tetracyclines was detected in some wild-type enterococci providing a cause for enterococci's intrinsic resistance (102). The *fmt* gene which affects the methicillin resistance level and autolysis was cloned and characteristized and suggested to be involved in cell wall synthesis (103).

UPDATE OF NEW ANTIBACTERIAL CLASSES

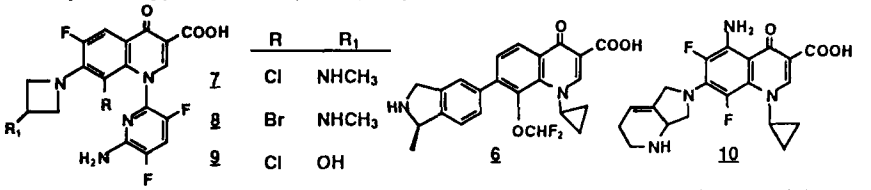
ß-Lactams - Reviews on pharmacology and antibacterial activity of penicillins and cephalosporins (104-106), ceftibutin (107), meropenem (108,109) and cefpirome (110)

have been published. A comprehensive review on catechol-type anti-pseudomonas cephalosporin appeared (111). New information on *in vitro/in vivo* activities or pharmacokinetics of previously reported carbapenem L-749,345 (112,113), CS-834 (114-117), the trinem GV 129606 (118), the antipseudomonal carbapenem ER-35786 (119), the anti- MRSA carbapenem BO-3482 (120,121) and the catechol-containing monobactam BMS-180680 (122) was published. The ß-lactamase inhibitory activity of Ro-48-1220(123) and other penam sulfones (124) was reported. Several papers on syntheses and structure-activity relationship of carbapenems (125-132) and CS-834 (133) appeared. Synthesis, *in vitro* and *in vivo* activities in combination with ß-lactams against B-lactamase producing strains of new potent ß-lactamase inhibitors azetidine sulfonic acid Syn 2190 (**1**) (134) and Syn-1012 (**2**) (135) were presented. A new cephalosporin MC-02,479 (**3**) was reported to have good *in vitro* and *in vivo* activities against multi-resistant gram-positive bacteria and have high affinity for PBP2a and stability against ß-lactamases (136). A series of 3-isoxazolylvinylcephalosporins exemplified by KST 150257 (**4**) was shown to have excellent *in vitro* activity against gram-positive bacterial including MRSA and good pharmacokinetics in mice (137). The *in vitro* activity, effect on alteration of porin and efflux pump of *P. aeruginosa*, clinical efficacies and side effect profile of the anti-pseudomonal parenteral carbapenem S-4661 were revealed (138). *In vivo* activity of peptidic prodrug of novel aminomethyl THF 1ß-methylcarbapenems was reported (139). Quinolonyl-lactams were found to possess potent antibacterial activity with good affinity for the PBPs. Although they are potent against MRSA, they do not bind to PBP2a (140). Among the several derivatives reported, PGE-7594630 (**5**) possesses the best overall activity both *in vitro* and *in vivo* (141). Structure-activity studies of quinolone-penems in genetically defined strains of *E. coli* has also been reported (142).

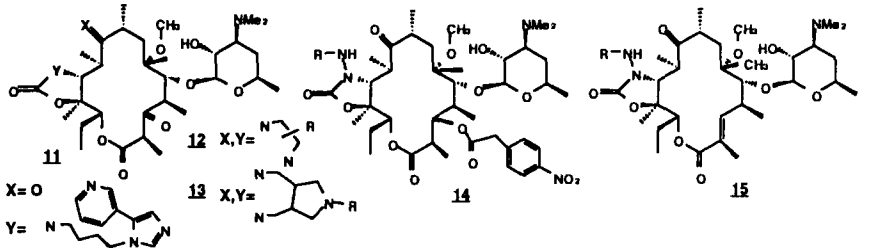


Quinolones - Comprehensive reviews on the antibacterial activity, pharmacokinetic properties and efficacy have been reported on sparfloxacin (143) and trovafloxacin (144, 145). Reviews on uses of recent quinolones were published (146-148). New information on *in vitro/in vivo* activities, pharmacokinetics, clinical efficacy and safety of previous reported quinolones trovafloxacin (149-158), BAY 12-8039 (159-165), DU-6895a (sitafloxacin) (166-169), HSR 903 (170,171), AM-1155 (gatifloxacin) (172-174), LB20304 (175,176), premafloxacin (177), CFC-222 (178), grepafloxacin (179) and Y-688 (180) were reported. T-3811 (**6**), a new 6-desfluoroquinolone, was reported to have excellent antibacterial activities including highly quinolone-resistant MRSA, *S. pneumoniae*, *S. pyogenes* and *E. faecalis*, (being 32-,16-,8- and 4- fold more active than ciprofloxacin). It also exhibited better safety profiles (181). WQ-2724 [**7**] and WQ-2743 (**8**) were shown to have better *in vivo* activity against *S. aureus*, MRSA, *S. pneumoniae* and *P. aeruginosa* murine systemic infections than tosufloxacin (182). A closely related analog WQ-3034 (**9**) possesses high activity against MRSA [MIC: 0.05 µg/ml at pH7 (50/12.5 µg/ml for ciprofloxacin/ sparfloxacin)] (183). The SAR on the 2-pyridone 8-substitutions was published (184). KRQ-10071 (**10**) was reported to have excellent antibacterial activity (MIC, µg/ml: 0.025, 0.013, 0.004, <0.002, 0.195, 0.007 and 0.007 vs *S. pyogenes*, *S. pneumoniae*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. typhimurium* and *E. cloacae*, respectively)(185). The result from a comparative study on quinolone-induced Achilles tendon toxicity on ten quinolones suggested that nitric

oxide and 5-lipoxigenase products partly mediate quinolone-induced tendon lesions (186). Chondrocyte toxicity and necrosis were seen with electron microscopy with ciprofloxacin or ofloxacin incubated human adult cartilage biopsy specimens (187). A recent study on the effect of 3-amino-1-hydroxy-2-pyrrolidone and gramma hydroxybutyric acid (GHB) by fluoroquinolones concluded that the excitatory adverse effects of quinolones might be treated by the administration of GHB (188). 6-Aminoquinolones possess good activity against *H. pylori* (189). The SAR study on N-1 t-butyl-substituted quinolones against *M. avium* appeared (190). Combination of trovafloxacin with amphotericin B or fluconazole was more effective in prolonging survival in murine model of invasive candidiasis than with either amphotericin B or fluconazole only (191). However, the mechanisms of action for this potentiation effect is not clear. A new method of assessing the bactericidal activity of quinolones and other antibacterial agents, known as bactericidal index was introduced (192). A few piperazinyloxoquinolines are potent and selective inhibitors of human immunodeficiency virus type 1 transcription (193).



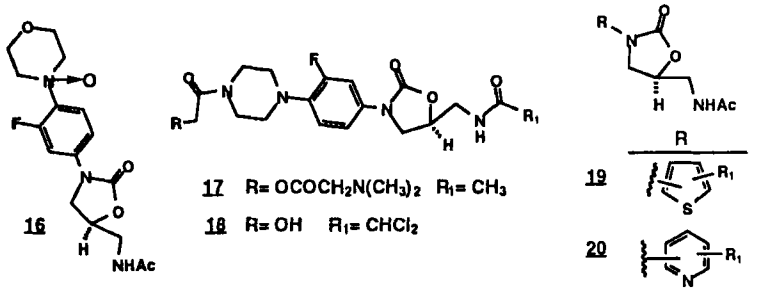
Macrolides - Previously reported ketolide RU 64004 is active against penicillin- and erythromycin-resistant pneumococci (MIC_{90}: 0.25 μg/ml) (194, 195). When tested against 379 anaerobes, it had an MIC_{50} of 1.0 μg/ml (196). Against 500 Gram positive organisms, including multiply resistant enterococci, streptococci and staphylococci, RU 64004 had a 100% susceptibility at a concentration of ≤ 1 μg/ml (197). It was strongly and rapidly accumulated by polymorphonuclear leukocytes (198) and was active against respiratory infections in animal study (199).



A closely related ketolide HMR 3647 (**11**) was as active as RU 64004 against anaerobes (200). HMR 3647 is also very active against *Toxoplasma gondii* (201). Many papers on the *in vitro* activity of HMR 3647 were presented at the 37th ICAAC (202). Tricyclic (**12**) and tetracyclic (**13**) ketolides were found to have potent antibacterial activity similar to RU 64004 (203). Other similar macrolides, acylides (**14**) and anhydrolides (**15**) were reported (204). New information on synercid (RP 59500) and an oral streptogramin RP 106972 related to *in vitro* activity, *in vivo* efficacies was reported (205, 206). Reviews on the *in vitro* and *in vivo* activities of quinupristin/dalfopristin have been published (207-209).

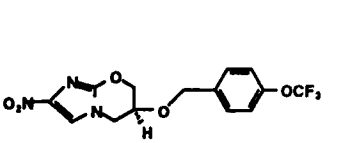
Glycopeptides - Reviews on the antibacterial activity, acquired resistance, pharmacokinetics and toxicity of glycopeptide antibiotics have been published (210-212). Additional information on *in vitro* antibacterial activity (213-216), *in vivo* efficacy (217) and pharmacodynamic properties (218,219) of the previous reported glycopeptide LY333328 appeared. Several carboxamide derivatives showed excellent activity against vancomycin-resistant and -sensitive enterococci, *S. aureus*, *S. hemolyticus*, *S. epidermidis* and *S. pneumoniae* (220). Several glycopeptide dimers were found to have improved activity against VRE over their respective parent glycopeptides (221).

Oxazolidinones - A review on the design and synthesis of novel oxazolidinones active against multidrug-resistant bacteria has been published (222). The *in vitro* activities (MIC_{90}) of oxazolidinone antibiotics eperezolid (U-100592) and linezolid (U-100766) against 100 isolates each of *S. aureus* and coagulase-negative Staphylococci were found to be 2 and 4 μg/ml, respectively (223). A new study of oxazolidinones concluded that the inhibition of bacterial translation at the initiation phase of protein synthesis may be the mechanism of antibacterial action (224). Although eperezolid binds to the 50S ribosomal subunit and competes with the binding of chloramphenicol and lincomycin, it acts by a mechanism distinct from those of chloramphenicol, lincomycin and clindamycin (225). N-oxide prodrug PNU-141535 [**16**] and ester prodrug PNU-101099 [**17**] are found to have increase water solubility and be converted to the parent oxazolidinones (226). The N-dichloroacetyl analog, PNU-109230 [**18**], displayed good activity *in vitro* with MICs against staphylococci, streptococci and enterococci of <0.5-2, <0.5, and 1 μg/ml , respectively (227). Novel phenyl-oxazolidinones containing saturated and 4,5-unsaturated 4-pyridinyl, pyranyl and thiopyranyl aryl substituents are found to exhibit increased activity against gram positives (228). Other novel oxazolidinones represented by **19** and **20** are found to have MICs ranging from 0.25 to 4 μg/ml against gram-positives with good *in vivo* activity (229).



Miscellaneous - Reviews on lipid A biosynthesis inhibitors as antibacterial agents (230), use of liposomes for controlled delivery and drug targeting (231), pharmacokinetics, pharmacodynamics, side effects, toxicity and interactions of aminoglycosides (232), use of inhibitors of bacterial virulence factors for infectious diseases therapeutic intervention (233), the methodologies of non-primate animal models of *H. pylori* infection (234), the mechanism of tuberculosis chemotherapy (235), the antibacterial activity, acquired resistance and clinical use of sulfonamides, rifamycins, nitrofurans, fosfomycin and comarins (236-240), and the determination of the therapeutic potential of experimental antibacterial agents by the use of *in vivo* animal models (241) have been published. The presence of C-1 amino group together with either an amino or hydroxyl group at the 2' and 6' positions of aminoglycoside antibiotics was found to be important for the inactivation of the antibiotics by enterococcal or staphylococcal aminoglycoside 3'-phosphotransferase type III a (242). L1,6-Anhydro-N-acetylmuramyl-L-alanyl-D-glutamyl-m-diaminopimelic acid -D-alanine was found to be the signal molecule for ß-lactamase induction in *E. cloacae* (243). Combination therapy for human brucellosis with doxycycline and gentamicin was shown to be effective (244). Recent study on tetracyclines indicated that tetracyclines were potentially useful for the prophylaxis and treatment of septic shock (245). A review on antimycobacterial agents appeared (246). A rapid screen for antimycobacterial activity by use of luciferase-expressing strain of *M. bovis* BCG and *M. intracellulare* (247) and low-dose aerosol infection model for testing drugs for efficacy against *M. tuberculosis* (248) have been established. The antimycobacterial activity and *in vitro* activity against *H. pylori* and toxicology of PA-824 (**21**) were reported (249). A novel bacterial two-component regulatory system inhibitor, RWJ-49815 (**22**) has activity against Gram-positives (MICs, 1-2 μg/ml) (250). Two potential antibacterial targets were mentioned: IMP dehydrogenase from *Pneumocystis carinii* (251) and dihydroneopterin aldolase (252). Reports on antibacterial peptides [protegrin-1 (253), bovine lactoferrin-derived peptides (254), and HIV type 1 derived peptides (255)], as

well as four plant essential oils (tea tree, lavender, mint and thyme) as antibacterial agents against *S. aureus* and VRE (MICs ranged from 0.25 to 1 μg/ml) (256) were reported.



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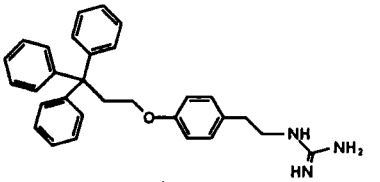

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Chapter 15. Recent Developments in Cancer Cytotoxics

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Introduction - The successful treatment of adult solid tumors remains a formidable challenge (1, 2). The partial success of traditional cancer chemotherapy stems in large part from agents derived from natural sources (3, 4). Cytotoxic agents, such as paclitaxel, CPT-11 and Topotecan, all of which have demonstrated significant clinical efficacy against a variety of human solid tumors, have revived interest in anticancer natural products research. The discovery paradigms employed in their identification have gone through evolutionary changes, from classical screening in tumor bearing animals, to today's sophisticated, disease-oriented multi cell panel screening approaches (5-8). In recent years, research efforts directed toward molecular target selective agents have proliferated. However, the clinical validity of these cellular targets remains to be established. Demonstrating proof of principle in the clinic may prove to be a greater challenge for these agents since theoretically they would produce tumor stasis rather than tumor regressions. Because the clinical validation of these new approaches is likely to be years away, cytotoxics will dominate the cancer chemotherapy scene for the foreseeable future. The aim of this report is to chart the progress of present day research efforts in the discovery of novel chemotherapeutics. Additional emphasis focuses on emerging future trends in novel cytotoxics and the selection criteria for their advancement to human clinical trials.

Traditional Cytotoxics - The majority of chemotherapeutic agents discovered prior to the 1980's were selected solely on the *in vivo* efficacy against leukemic (P388 and L1210) tumor models (9). The lack of appropriate human and solid tumor efficacy models resulted in the discovery of numerous agents endowed with a limited range of mechanisms with meager activity vs. solid tumors and a narrow therapeutic index. The long development history of paclitaxel is a classical example of the preclinical and clinical hurdles which can be encountered by a drug enroute to clinical success. To date, efforts by medicinal chemists to improve upon the efficacy and toxicity profiles of many natural chemotherapetic agents such as mitomycin C and doxorubicin have met with very limited success. However, there are examples where the side effect profile of a clinically used cytotoxic has been improved upon by an analog. Carboplatin is one such success story. It is equiactive to cis-platinum but endowed with less renal and neurotoxicity. As discussed in sections below, R&D efforts are continuing in all categories of chemotherapeutic cytotoxics to discover drugs with a broader spectrum of activity and less toxic side effects. The desired spectrum of activity of new agents is focused towards the treatment of more common solid neoplasms such as lung, colon and breast cancers. Also, attempts to combat the emergence of induced and innate drug resistance in the clinical setting are being undertaken at various levels (10).

ANTIMITOTIC AGENTS

Tubulin stablizing agents - The taxane class of antimicrotubule anticancer agents have become the most important addition to the chemotherapeutic armamentarium against cancer (11, 12). The two clinically utizilized taxanes, paclitaxel **1**, the active ingredient of TAXOL®, and docetaxel **2** (TAXOTERE®), have consistently shown impressive activity in advanced cancers of the ovary, breast, lung, esophagus, bladder, and head and neck. Even with this impressive anticancer activity and clinical success, considerable efforts to enhance efficacy, improve activity against resistant tumors, or improve the pharmaceutical properties such as water solubility, of the clinical taxanes have been described in recent years. To this end, there has been an intensive effort to design taxane analogs that can build upon the success of TAXOL® and TAXOTERE®.

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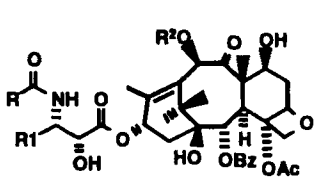

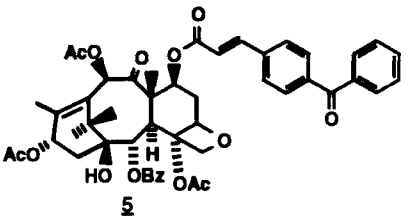

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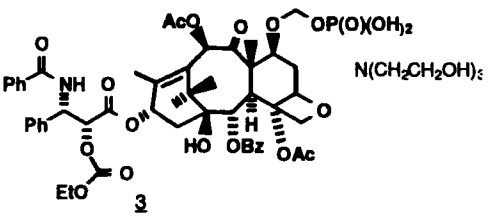
	R	R^1	R^2
1	Ph	Ph	Ac-
2	Ph	Ph	H
4	tBuO-		Ac-



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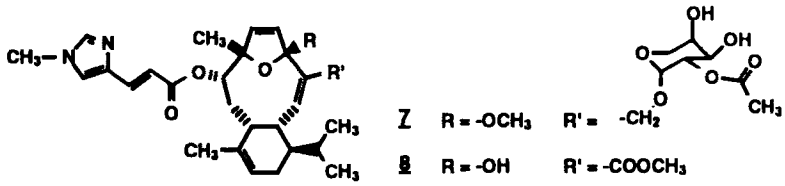
The solution and solid state conformations of the clinical taxanes have been well studied (13-18). Recently a 3.7Å resolution electron microscopy structure of paclitaxel stabilized microtubules has recently been reported, although the bound conformation of paclitaxel was not identified (19). A number of reviews on the taxane field have been published (20-22). Analogs of the clinical taxanes with modifications at almost every position have been reported. Many of these compounds retain or even possess improved potency against *in vitro* cell lines (23-31). A potential advantage of a water soluble taxane for iv administration is that the use of possibly toxic, solubilizing excipients would be minimized (32). Water soluble prodrugs such as BMS-185660 (3) were identified and found to be efficacious against a variety of tumors implanted in host mice (33). However, it was later found to be non toxic in higher, non rodent species due to lack of hydrolysis of the 2' ethyl carbonate on the sidechain hydroxy group (34). Both paclitaxel and docetaxel are substrates for p-glycoprotein or multiple drug resistance. A number of reports of taxane analogs with superior activity against taxane resistant cell lines have appeared (34, 36, 41). SB-T-1213 (4) is an example of a taxane analog which possesses greater *in vitro* potency and shows nearly completely non cross resistant against an adriamycin resistant cell line (30). SB-T-1213 was quite active against a taxane sensitive subcutaneous B-16 solid tumor model. Athough it was more potent than docetaxel, it was less active when compared at optimum doses. The activity of this compound and similar analogs is being evaluated in resistant tumor models. The affinity of the taxane structure for p-glycoprotein is illustrated by a report that non toxic 10-desacetyl baccatin derivatives such as 5 have been found to be potent MDR reversal agents *in vitro* (35). Clinical studies are underway with paclitaxel in combination with several different MDR modulaters including cyclosporine, verapamil, or VXR-710 with the aim of providing increased efficacy against resistant tumors (36-39). Two papers have described the increased oral bioavailability of paclitaxel in mice when coadministered with either

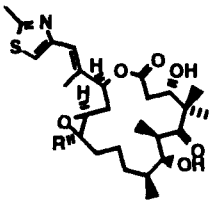


7 R = -OCH_3 R' = -CH_2

8 R = -OH R' = -$COOCH_3$

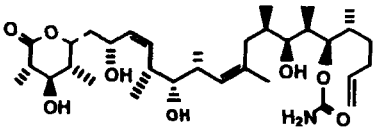
cyclosporine or a cyclosporine analog PSC-833 (40-42). Several patent applications covering the use of MDR inhibitors and an oral taxane have published (40, 43-45). An oral taxane may provide a means to achieve prolonged tumor exposure to this effective class of anticancer agent and thereby improve its activity against resistant tumors.

A number of non taxane cytotoxic, tubulin polymerization agents have been identified in recent years. Discodermolide (6), eleutherobin (7), and sarcodictyin A (8) are marine natual products which accelerate tubulin polymerization in a manner similar to that of paclitaxel (46-50). Whereas discodermolide appears to be more potent than the taxanes in tubulin polymerization assays (46, 47), the eleutherobins and sarcodictyins are nearly equipotent to paclitaxel (48, 49). However, in cytoxicity assays, eleutherobin is about half as potent as paclitaxel while the sarcodictyins are considerably less potent (48, 49). Eleutherobin is cross resistant to paclitaxel resistant cell lines (48, 51). The eleutherobins, sarcodictyins, and epothilones (described below) were found to be competitive inhibitors of tritiated paclitaxel in tubulin binding studies (48, 49, 52). Syntheses of sarcodictyin A and eleutherobin have been published (53, 54). The epothilones such as epothilone A (9) or



9 R = -H

10 R = -CH_3



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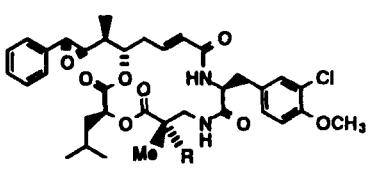
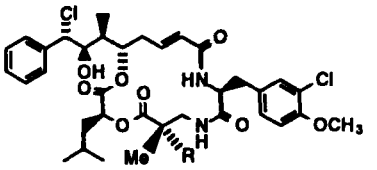
epothilone B (10) are microbial-derived tubulin polymerizing natural products that can be obtained from fermentation (55). In addition, the epothilones possess greater water solubility than the two clinical taxanes (56). Epothilone B exhibits potency similar to paclitaxel in tubulin polymerization and cytotoxicity assays (52, 57). One potential advantage of this class of compounds is their lack of cross resistance to paclitaxel in a number of resistant cell lines (58). The epothilones have yielded to total synthesis and combinatorial and solid phase analog syntheses have also been published (59-67). Considerable *in vitro* SAR has been developed on these synthetic analogs (61, 68).

Microtubule Formation Inhibitors - The clinically approved tubulin polymerization inhibitors are all vinca alkaloids. Vinorelbine is a highly active compound which is likely to continue to increase in clinical importance (69). F 12158, 20'20'-difluoro-dihydrovinorelbine, is a novel, semisynthetic vinca selected for futher examination based on its slightly improved *in vivo* antitumor activity over vinorelbine (70). The tubulin depolymerizing cyclic peptides, dolastatin 10 and dolastatin 15, are in Phase I trials and preclinical development in Japan, respectively (71, 72). Recently two new cytotoxic compounds dolostatin 16 and dolastatin 17 have been reported (71, 73). The halichondrins such as halichondrin B have been the object of considerable synthetic effort due to their activity in preclinical models (74). The cryptophycins are a relatively new class of microtubule inhibitors which were originally isolated in 1994 from cyanobacteria (75). Cryptophycin I (11) is a potent inhibitor of cell proliferation and like the vinca alkaloids causes the loss of cellular microtubules (76, 77). Cryptophycin 1 is a considerably more potent inhibitor of microtubule dynamics than paclitaxel or vinca alkaloids and detailed *in virtro* studies of mechanism have been published. It's ability to rapidly initiate apopotosis has been suggested to be partially responsible for the observed cytoxic effects (78). Compound 12, C-52 (LY355702) and compound 13, C-55 (LY355703) are synthetic, gem-dimethyl analogs of cryptophycin 1. C-52 is the chlorohydrin of C-55 and is converted to C-55 *in vivo* (79). The compounds have been shown to have no cross resistance to adriamycin or paclitaxel *in vitro* (80). In human tumor xenograft models the maximum tolerated dose of C-52 is approximately 5-fold higher than that of C-55 (79). Against a panel of human tumor xenograft models, both compounds exhibited 90-100% tumor growth inhibition and C-52 has been advanced to clinical development (79-81).

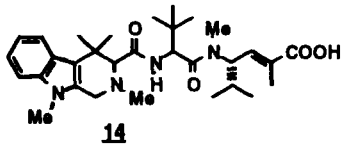


11 R = -H
13 R = -Me



12 R = -Me

The naturally occurring milnamide A (14), hemiasterlin (15), hemiasterlin A (16), and criamide (17) are members of a small family of tri- and tetrapeptides containing two highly modified amino acids which are isolated from marine sponges (82). These compounds inhibit microtubule formation by binding to the vinca alkaloid site on tubulin. They show potent *in vitro* activity against human breast, ovarian,



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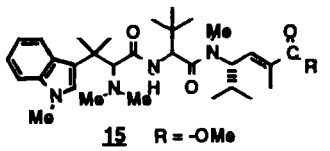
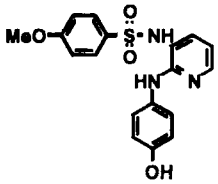


15 R = -OMe
16 R = -OH
17 R = -NH-Arg



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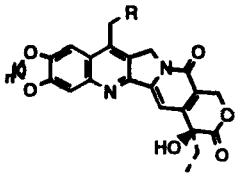
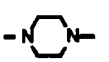
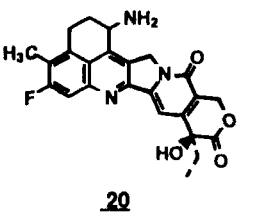
colon, and lung cancer cell lines. A total synthesis of (-) hemiasterlin has been published (83). Few compounds which bind to the colchicine binding site of tubulin have activity against *in vivo* solid tumor models. However E7010 (18), an orally active sulfonamide, inhibits mitosis by binding to the colchicine binding site and possesses significant activity against solid tumors (84). In addition, the compound appears to be non cross resistant to vincristine or paclitaxel in many resistant cell lines.

Topoisomerase Active Agents - Two water soluble semisynthetic camptothecins, topotecan (85) and irinotecan (86), entered the anticancer drug arena as topoisomerase-I active agents during the last two years. Despite their structural similarity, they both differ in their antitumor spectrum of activity as well as in their side effect profiles (85, 86). Irinotecan is approved for colorectal cancer and severe diarrhea associated GI toxicity is the dose-limiting toxicity (DLT), while the former suffers from neutrophenia as DLT and is approved for ovarian and small cell lung carcinoma. Several recent reports review this class of agents (87, 88). Some of these newer camptothecin analogs are currently in clinical trials (89). These include the water soluble analogs GI 147211C (19) , 9-aminocamptothecin (9AC), and the hexacyclic DX-8951F (20) (90). Several analogs of 19 were published (91) and the quarternary ammonium salts 21 and 22 were more efficacious than topotecan in delaying tumor growth in HT-29 human xenograft tumor models (92). Analog 22, in an extended *in vivo* protocol, demonstrated tumor regression. Structure-activity studies on the hexacyclic camptothecin analogs are also reported and generally this ring appendage results in a conformational rigidity at the 7-position of topotecan and causes a favorable antitumor activity profile. DX-8951F, a recent entry to the clinic, is claimed to possess activity in a broder spectrum of xenograft models as well as against p-glycoprotein mediated drug resistant cell lines (90).



Compound	n	R
19	2	
21	1	
22	2	



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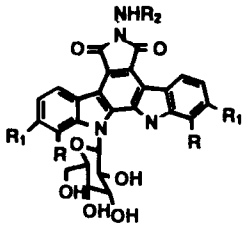
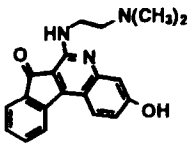
Beyond the camptothecin analogs (93-97), certain indolocarbazole agents have also entered the clinic and their presumed mechanism of action is claimed to be inhihibition of topoisomerase I (98). Other mechanisms such as RNA polymerase activity and topo-II activity have been suggested for these compounds (99). NB-506 (**23**), the first generation compound inhibited the solid tumor growth of murine and human solid tumors in preclinical models (100, 101). This agent shows strong synergy with cisplatin *in vitro* against human small cell lung cancer cells, suggesting the utility of such combination therapy. Currently, a back up compound, ED-749 (**24**), is entering Phase I clinical trials.



23 R = OH, R_1 = H, R_2 = CHO

24 R = H, R_1 = OH, R_2 = CH $(CH_2OH)_2$



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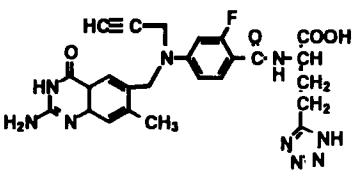
Topoisomerase II active agents enjoyed considerable attention during the early 80's and resulted in clinically active agents. In contrast to the rich literature of topo-I (102, 103) and topo-II active agents (104, 105), much less is reported on the dual active agents (106). The rationale for this approach stems from the fact that the levels of the two enzymes and time course of expression differ. Also, the expression of either enzyme appears to be sufficient to support cell division, and the development of resistance to topo-I active agents is often accompanied by a concomitant rise in the level of topo II and *vice versa.* Sanitopin, benzophenanthridine alkaloids coralyne nitidine, fagaronine, acridine, anthracenyl dipeptides, bisnapthylamides, certain pridoindole and indolocarbazoles and indenoquinolone show this dual mechanism of action. However, only two compounds known to stimulate both topo-I and topo II mediated DNA cleavage, intoplicine, mentioned in Volume 28 of this series, and TAS-103, are currently in clinical trials (107). Reports that intoplicine caused liver toxicity have resulted in a change in the protocol to a prolonged continuous infusion in order to circumvent this toxicity. It has been suggested that toxicity to normal tissues may result from the use of a sequential combination therapy of topo-I and topo-II agents (108).

Antimetabolites - Several detailed reviews on the status of antifolates in clinical development have recently appeared (109-112). These describe in detail the more than 10 antifolate compounds which are currently being evaluated clinically as well as several compounds which appear poised to progress to the clinical setting. Although many of the compounds in development may appear structurally similar to methotrexate, the most widely used antifolate, their properties can differ considerably. The new compounds may have specifity for different enzyme targets [thymidylate synthase (TS), dihydrofoalte reductase (DHFR) or glycinamide ribonucleotide formyl transferase (GARFT)], may possess different membrane transport properties, or differ in their ability to undergo intracellular polyglutamylation (109). Several compounds reported in Volume 28 of this series have made significant progress. Trimetrexate (TMTX) has been shown to be particularly useful in combination with 5-FU (5-fluoro uracil) and leucovorin (LV) for the treatment of colorectal cancer (CRC) (109, 111). Current studies are aimed at assessing its utility in gastric cancers either as a single agent or in combinations. Piretrixin is in Phase I clinical trials (109). Like TMTX, piretrexim lacks a glutamic acid sidechain and thus is not polyglutamylated in the cell. As a consequence, both compounds are not retained in the cells for long duration despite initially high intracellular concentrations. Like TMTX, piretrexin is less effective against cells expressing the MDR phenotype. The principal advantages of piretrexin over TMTX are its oral bioavailability and lack of effect on histamine metabolizing enzymes (109). Oral piretrexin has seen less clinical

use than other antifolates but its best activity has been for treatment of transitional cell carcinoma of the bladder. AG 337 showed minimal activity in Phase II trials and the deveopment was terminated (109). Phase I trials of AG 331 were halted by hepatoxicity, fever and overall drug intolerance (109). Edatrexate is in Phase III clinical trials but so far has not demonstrated any advantage over methotrexate or standard therapies against various tumors (109). Lomotrexol is a specific GARFT inhibitor that showed severe cumulative myelopsuppression in Phase I trials and this has halted Phase I testing (109). LY231514 was originally thought to be a pure TS inhibitor but was later determined to also inhibit purine synthesis and DHFR. Preliminary reports from Phase II trials have described encouraging response rates in non small cell lung (NSCLC), breast, and CRC patients. CRC patients previously treated with 5-FU did not develop cross resistance to LY231514. Ongoing clinical trials are designed to define its role in previously treated CRC patients (109). Raltritrexed is in Phase II trials and is a promising drug for advanced CRC because it can produce similar antitumor response rates than 5-FU/leucovorin but with lower toxicity. 1843U89 is the most potent inhibitor of TS in clinical trials. Early clinical studies determined that it must be coadministered with oral folic acid to suppress side effects but further studies are still planned (109). ZD 9331 (**26**), a water soluble



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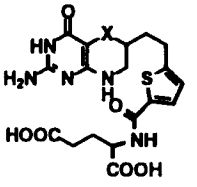
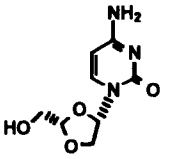


27 X = -S-
28 X = -CH_2-

drivative of raltritrexed, is a pure TS inhibitor that is in Phase I trials. AG2034 (**27**) is a rationally designed specific inhibitor of GARFT which has just started Phase I trials. LY309887 (**28**) is a thiophene analog of lometrexol which is a more potent GARTF inhibitor and is now entering Phase I clinical studies (112). Tomudex, a thymidylate synthesis inhibitor, is currently in Phase III clinical trials (118).



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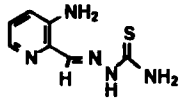

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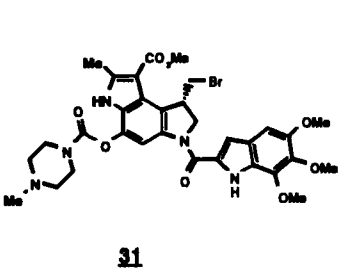
The novel nucleoside analog BCH-4556 (**29**) shows good activity against human renal cell xenografts as well as many other solid tumor models (113). This compound is possibly the only unnatural anticancer nucleoside with the sugar in the β-D-configuration. It is a potent chain terminator, and unlike ARA-C is resistant to deactivation by deoxycytidine deaminase. In addition, when incorporated into DNA, BCH-4556 causes instant chain termination while incorporation of Ara-C can support continued DNA synthesis. BCH-4556 has been recommended for clinical development (113).

Ribonucleotide reductase is a critical enzyme for DNA synthesis since it catalyzes the rate limiting step of deoxyribonucleoside triphosphates in all cells (114). The presence and activity of this enzyme correlate closely with cell growth rates (115, 116). Among the known inhibitors, only hydroxyurea and gemcitabine (GEMZAR®) are approved for clinical use (114). Gemcitabine was approved for use in the U.S. in 1996 and Phase III clinical studies for bladder, breast, non small cell lung cancers are in

progress (117). It's utility against other cancers and in combinations is also under investigation. MDL-101731 is in Phase I studies against solid tumors in Europe and Japan (118). Another ribonucleotide reductase inhibitor selected to enter clinical trials based on preclinical (114) *in vivo* antitumor efficacy is 3-AP (**30**) (114).

A number of 5-FU prodrugs have been synthesized in the last decade in an effort to improve therapeutic index and minimize side effects(112). The best known example is 1-(tetrahydro-2-furanyl)-5-fluorouracil (tegafur)(112). This compound is currently in clinical trials as an oral formulation with uracil as UFT. The uracil prevents the enymatic degradation of 5-FU by cellular enzymes and increases oral bioavailability. S-1 is a combination designed to improve the efficacy of UFT even further (112). It contains an enzyme inhibitor with greater potency than uracil and oronic acid which is added to reduce gut toxicity. Oronic acid concentrates selectively in the gut and is an inhibitor of oroate phosphoribosyltransferase, an enzyme necessary for the activation of 5-FU. A carbamate prodrug of 5-FU, capecitabine produced responses in Phase I trials after oral dosing (69). This compound is metabolized to 5-FU in the liver after absorption which minimizes side effects to healthy tissues and FDA approval for use in refractory breast cancer is pending.

DNA Interactive Agents - Analogs of CC-1065 were discussed in Volume 28 of this series. The delayed toxicity exhibited by CC-1065 is not observed in compounds with either altered DNA recognition or which exhibit reversible, rather than covalent DNA alkylation. A number of analogs with one or both of these properties remain of interest either clinically or preclinically. Bizelsesin is currently in Phase I clinical trials (119). Carzelesin has almost completed Phase II clinical trials against a panel of seven solid tumors (119). The related adozolesin completed Phase II trials but has not entered Phase III. (+) Duocarmycin SA is a natural product produced by *Streptomyces sp.* KW-2189 (**31**) is a duocarmycin SA analog with greater antitumor activity in preclinical *in vivo* models and better water solubility than the parent (120-122). This analog is currently undergoing clinical trials. This analog is proposed to alkylate DNA without loss of the carbamoyl moiety. Efforts to enhance the properties of duocarmycin have appeared in the literature (123). Seco-(+)-Oxa duocarmycin SA (LY307918) (**32**) as well as its enantiomer are being evaluated preclinically based on initial promising data (124). The fused pyrrole ring in the alkylating subunit is replaced with a potentially more metabolically inert furan ring. Doxorubicin and daunorubicin were the most clinically useful of the first generation anthracyclines introduced in the early 70s. Doxorubicin, the most active of the two, remains an important clinical agent at present. Extensive analog programs failed to produce analogs with greater efficacy against solid tumors but did produce the better tolerated epirubicin and a highly potent antileukemic drug idarubicin. Recent synthetic efforts on disaccharide analogs have culminated in the identification of a third generation anthracycline, MEN 10755 (**33**), a compound which appeared superior to doxorubicin in inhibiting tumor growth in preclinical models. MEN 10755 is currently undergoing Phase I clinical trials in Europe (125). Initial Phase I studies on a liposomal formation of daunorubicin have been completed and more advanced evaluation was recommended (126). Some responses were observed. The bis-naphthilimide intercalator DMP 840 displayed activity against human tumor xenograft tumors in mice and is currently in Phase II clinical trials (127). DMP 315 is a potent, water soluble antitumor agent which has been advanced for further study (127). The anthrapyrazole, losaxantrone has utility in the the treatment of breast cancer and also appears to be less cardiotoxic than doxorubicin (69). New platinum analogs (oxaliplatin, JM-216) are still in early stages of development and are undergoing clinical studies in Europe (69). Both show incomplete cross resistance with cisplatin. The DNA cleaving properties of the novel antitumor antibiotic leinamycin (**34**) were determined to be mediated by thiol dependent formation of radicals from molecular oxygen (128).



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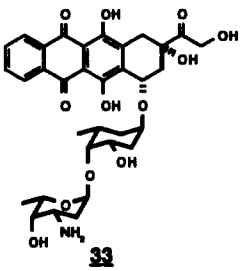

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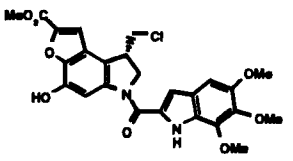

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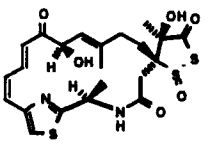

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Miscellaneous - Tumor selective/targeted therapies include certain drug delivery systems such as simple prodrugs (129, 130), antibody directed enzyme prodrug therapy (131), polymer conjugates, and combination therapy with multidrug resistance reversing (MDR) agents (10, 132, 133). A recent report of targeted delivery of a cytotoxic agent such as maytansine via its folic acid conjugate to folic acid positive receptor cells has appeared (134). The authors' premise for this effort was based on the fact that cancerous cells over express folic acid receptors in comparison to their normal counterparts and thus could potentially provide drug targeting opportunities via the folic acid transport mechanism. Several recent reviews address some of the issues and advances made in area of tumor selective therapy. In this context, a recent report shows the potential for using phage display techniques to identify certain small peptides that are specific to angiogenic vessels and then linking these peptides to a cytotoxic drug such as doxorubicin for tumor selective therapy (135). A number of new natural products with unknowm mechanisms have been disclosed (136).

Next Generation Chemotherapeutics - The future scenerio in clinical management of cancer will be mainly dictated by the availability of less toxic and tumor selective agents (137). Their optimal use may be as single agents or in combination with traditional cytotoxic agents with the treatment goal of imparting cures and not just palliation. These efforts will be further aided by the increased understanding of oncogene signaling pathways, cell cycle targets (136) and angiogenesis (138, 139). Gene therapy as well as several emerging or recently covered targets are not discussed here and include telomerase (140), growth factors (141, 142), cyclin dependent kinases, and RAS/FT inhibitors (143-146). Non-receptor tyrosine kinases (147, 148) have been identified as attractive drug discovery targets. Recent examples (70) of non toxic cytostatic kinase inhibitors moving towards clinical trial include CP-358,774, ZD 1839, and CEP-751. Combination therapy which involves the use of a cytotoxic and either a cytostatic or an angiogenic agent might be the cornerstone for future chemotherapy. Furthermore, improved diagnosis and increased use of orally active anti-cancer agents would set the stage for early intervention and more outpatient care.

Future therapies tailored to the genetics of the tumor and the patient will have an enormous impact on treatment outcome 149). For example, the most common mutation in cancer is the p53 tumor suppressor gene. Mutations convey resistance to DNA active cytotoxics such as cisplatin and 5-FU. Age and population related changes in the levels of cytochrome P450 and other drug metabolising enzymes may also effect drug levels and therefore therapeutic response (150). The revolution in

gene- based diagnostics will lead to earlier detection and the identification of patients at risk of developing cancer and define a critical need for safer chemopreventive strategies.

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Chapter 16. Advances in Nucleoside and Nucleotide Antiviral Therapies

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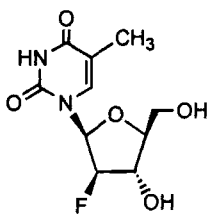
Introduction - Nucleoside therapy continues to be the cornerstone for treatment of viral diseases, particularly those associated with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and herpes simplex virus (HSV). Several recent reviews summarizing nucleoside antiviral research were published (1-4). Significant clinical advances with carbocyclic and L-nucleosides were made during the past two years. Preclinically, there are a number of promising L-nucleosides under study and major advances in the arena of delivering nucleotides ('protides') into cells were reported. This chapter includes advances over the past two years on nucleoside analogs that are in, or appear to have the best chance of entering clinical trials.



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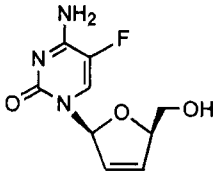

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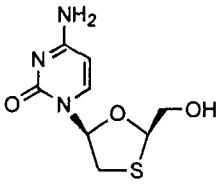

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D- and L- Furanosides - Several L-pyrimidines, (1-3) progressed in preclinical studies as potent chemotheraputic agents against HIV and HBV. L-FMAU (1) was confirmed to be the most potent of a series of pyrimidine analogs with anti-HBV activity (IC_{50} 0.1 μM). 1 was also active against Epstein-Barr virus (EBV) (5). Preclinical investigations of 1 included a 30-day toxicity study in mice. Doses of 10 or 50 mg/kg/d showed no significant toxicity. The pharmacokinetics of 1 was described (6) along with *in vivo* efficacy in a woodchuck hepatitis virus (WHV) carrier model. Treatment for 12 weeks at 10 mg/kg/d a gave greater than 1000-fold reduction in serum WHV DNA levels. Levels of virus in three of four treated animals remained undetectable for more than 36 weeks (7).

β-L-Fd4C (2) was potent and selective against both HBV and HIV (IC_{50}, 0.002 μM and 0.1 μM, respectively). 2 did not affect mitochondrial DNA synthesis at 20 μM and favorably potentiated the anti-HIV activity of AZT and d4T. High C_{max} values (150 μM) were achieved upon dosing mice for 30d at 100 mg/kg/d (po). No evidence of toxicity was observed (8).

The clinical development of lamivudine (3TC) (3) for the treatment of HBV continued. Clinical results indicated a consistent, marked inhibitory effect on HBV replication. Patients consistently exhibited multi-log reductions in serum HBV DNA

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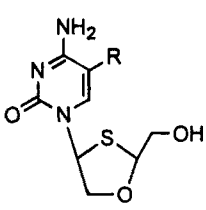
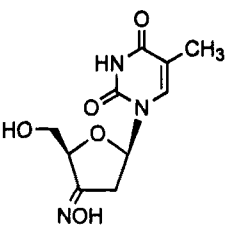
after 1 to 4 weeks of treatment (9). Studies in woodchuck hepatitis virus (WHV) infected woodchucks suggest that treatment with 3 delays the development of hepatocellular carcinoma (10). Racemic OTC (4), a positional isomer of 3, and its 5-fluoro congener (5) appear to be potent anti-HIV agents (IC_{50}, 0.1 to 3 μM) (11). Surprisingly, these analogs retain significant activity against 3TC-resistant HIV (11).

A novel 3'-N-hydroximino analog of thymidine (6) was reported to block replication of HIV-1 and -2, HSV, and HBV *in vitro*. Although the mechanism of action is unknown, in HSV infected cells anabolism to the monophosphate by the viral thymidine kinase (TK) is strongly suggested (12).



4 R = H
5 R = F



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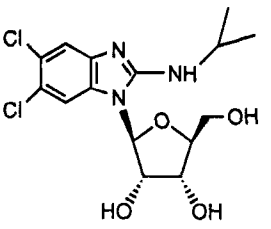

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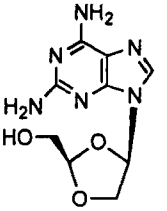
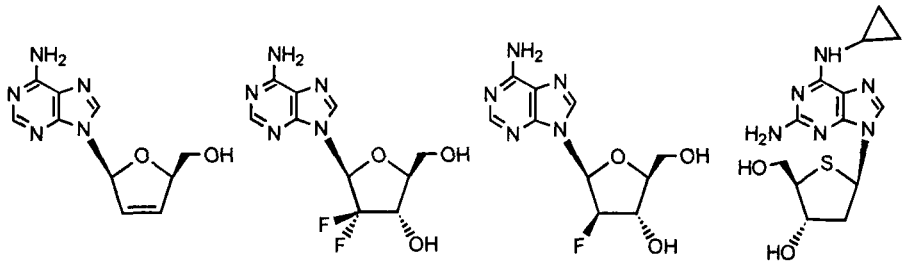


8

A variety of purine nucleosides and congeners are at various stages of development. The L-benzimidazole riboside, 1263W94 (7), was well-tolerated with plasma levels well above the *in vitro* anti-HCMV IC_{50} (0.1 μM) in a Phase 1 study in HIV-infected individuals (13). Preclinical development of (-)-β-D-diaminopurine dioxolane (8), a prodrug of the guanine analog, continued. Against HBV, 8 had an IC_{50} of 0.01 μM and in combination with either 3 or interferon was reported to give a synergistic response. In a three month *in vivo* woodchuck study, 8 at 1 mg/kg/d reduced the level of WHV serum DNA by greater than 1 log. No toxicity toward the animals was reported (14).

Various sugar-modified β-L-adenosines were also described that inhibit HBV replication. β-L-ddA, 2'-azido-β-L-ddA, 3'-azido-β-L-ddA and 2'3'-didehydro-β-L-ddA (L-d4A) (9) all blocked HBV replication at 5 μM or less. The most potent analog in this series was 9 (IC_{50} 0.1 μM) which also exhibited significant anti-HIV activity (IC_{50} 0.4 μM) (15). A series of purine and pyrimidine 2',2'difluoro-L-nucleosides were prepared. Only the adenine analog (10) had anti-HIV activity (IC_{50} 3.4 μM) (16). The 2'-monofluoro arabinoside analog (11), exhibited good *in vitro* anti-HBV activity (IC_{50} 1.5 μM) (17).

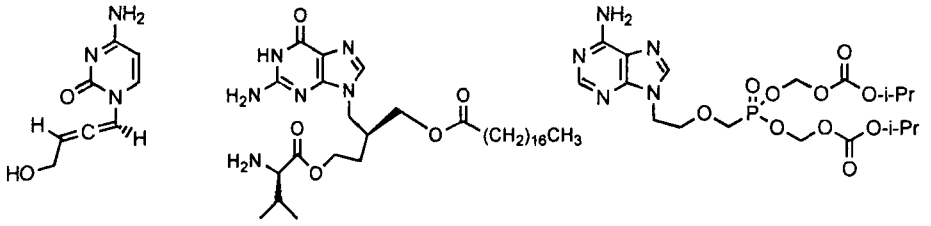
4'-Thionucleosides continue to be explored because of their structural similarity to 4'-oxo-nucleosides and their enhanced stability towards phosphorolytic enzymes. 2'-Deoxy- (18-21) and ara-4'-thionucleosides (22,23) have been prepared in sufficient quantity to allow *in vivo* characterization. In general, the β-D-analogs exhibit modest to potent anti-herpetic activity *in vitro*, yet most lack a suitable selectivity index for therapeutic use. Of particular note, the 2'-deoxy-4'-thio diaminopurine analog, 12, was found to be potent against HCMV (0.2 μM) and HBV (0.007 μM), but exhibited severe kidney and liver toxicity in the beagle dog (18).



9 10 11 12

Acyclic Nucleosides - Preclinical and clinical summaries have recently appeared (24-26). Oral strategies for ganciclovir (GCV) delivery have not demonstrated marked efficacy improvement over intravenous ganciclovir delivery for HCMV retinitis (27). Recent clinical studies using PCR technology indicate that GCV resistance occurs more rapidly and frequently than previously demonstrated with phenotypic assays (28,29). Investigations of the anti-HBV activity of penciclovir and its 6-deoxy diacetyl prodrug, famvir, have reported synergism with lamivudine (30) and dose-dependent reduction of HBV DNA (31). Anti-HBV activity for the (R)-enantiomer of cytallene (IC_{50} 0.05 μM) (13) has been noted and it has been suggested that racemic (R,S)-cytallene may have therapeutic benefit (32). Replacement of the allene double bond distal from the base with a cyclopropane ring has provided analogs with anti-herpetic and anti-HBV activities in the low micromolar and sub-micromolar range (33).

ABT-606 (14) was selected as an orally bioavailable diester prodrug of the broad spectrum anti-herpetic H2G. HSV-1 clinical trials are in progress (34).

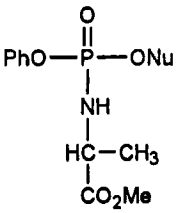


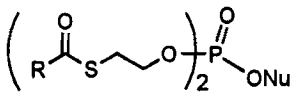
13 14 15

Acyclic phosphonates and their prodrugs continue to be of interest in antiviral therapy. An orally bioavailable prodrug of the anti-HIV agent PMPA, bis-POC PMPA (15), was selected as a clinical candidate from a variety of carbonate and carbamate prodrugs (35).

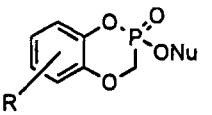
Nucleotide Prodrugs - Efforts to effect intracellular delivery of nucleotides have made substantial progress in the last two years. In specific instances, efficient kinase bypass has been accompanied by impressive increases in potency, lessened cytotoxicity, and lengthened half-life of intracellular nucleotides. These advances may result in improved therapeutic agents and have special implications for dealing with the emergence of resistance to nucleoside antivirals. However, as noted in a recent

review (36), the complex metabolism, excretion, and tissue distribution of nucleotide prodrugs makes extrapolation from *in vitro* assays to *in vivo* efficacy difficult.





(R = Me, i-Pr, t-Bu)



16 17 18

Nu = 5'-O-substituted nucleosides: d4T (16a, 18a); ddA (16b, 17b, 18b), d4A (16c), iso-ddA (16d, 17d), PMEA (17e), AZT (17f), ACV (17g), L-ddA (17h).

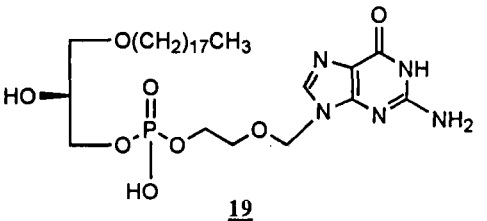
Recent *in vitro* successes have been reported with (aryloxy)phosphoramidates (16), bis-S-acyl-2-thioethyl (SATE) esters (17), and dioxaphosphorine oxides (18) (37-41).

Applying the (aryloxy)phosphoramidate approach, the anti-HIV activity of 16a was improved 4- to 10-fold over that of d4T. In contrast to d4T, 16a inhibited HIV replication in thymidine kinase-deficient cells, consistent with delivery of the monophosphate and kinase bypass (42,43). The amino acid is an important determinant of potency and L-alanine appears to be optimal (44-46). Understandably, no improvement in potency was noted when this approach was applied to nucleosides for which the first phosphorylation step to monophosphate is not rate-determining (AZT and ddI). The phosphodiester alaninates of ddA (2',3'-dideoxyadenosine) (16b) and d4A (2',3'-didehydro-2',3'-dideoxyadenosine) (16c) rank among the most potent HIV and HBV inhibitors reported to date (47, 48). Aryl substitution has been reported to be unnecessary with certain phosphoramidates containing lipophilic amino acids (49).

The acyloxy groups of bis-S-acyl-2-thioethyl (SATE) esters (17) are cleaved by carboxyesterases and the resulting intermediates then decompose spontaneously to episulfide and the nucleoside-monophosphate. The t-butyl SATE esters typically have greater stability in human serum and cell extracts than the methyl analogs. The decomposition kinetics of the iso-ddA analog 17d has been compared to that of the corresponding (aryloxy)phosphoramidate 16d in CEM cell extracts (50). The bis(t-Bu-SATE) derivative of 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) 17e had comparable *in vitro* anti-HIV potency and greater stability in gastric fluid and serum than the bis-POM ester, clinical candidate adefovir dipivoxil (51). No additional toxicity due to episulfide generation was evident by evaluation of 17f, the AZT SATE derivative with R = methyl, in human bone marrow progenitor cells in culture (52). Bis(methyl-SATE) ddA (17b) was 3- to 4-logs more potent than ddA against HIV (53), paralleling the excellent potency of the phosphoramidate analogs. Although ACV is inactive against HBV, SATE derivatives 17g (methyl and t-butyl) were as active as ddG (IC_{50} 1-3 μM) (53, 54). The bis(t-butyl)SATE derivative of L-ddA (17h) was recently reported to be an extremely potent inhibitor of HBV (IC_{50} < 0.1 μM) with marked *in vitro* synergy with 3TC (55).

The cyclosaligenyl (Cyclo-Sal) esters of nucleoside monophosphates (**18**) were shown to be cleaved to the monophosphates and salicylalcohols by a controlled, chemically-induced hydrolysis involving a successive coupled cleavage of the phenyl- and benzyl-esters (tandem mechanism). A correlation has been demonstrated between the electronic properties of the salicylalcohol substituents (R) and the hydrolysis half-life of the triester (41). Activity of the d4T analogs **18a** in thymidine kinase deficient cells confirmed kinase bypass (56). The ddA analog **18b** is 100-fold more potent than ddA against HIV (57).

Other approaches to nucleotide delivery continue to receive attention. Promising *in vivo* results were reported for lipid conjugates of nucleoside monophosphates. Enhanced *in vitro* anti-HBV activity and improved oral delivery of ACV in mice was seen with 1-O-octadecyl-sn-glycero-3-phospho-acyclovir (**19**) and related lipid diester prodrugs of ACV-monophosphate (58). Simpler long chain alkoxy and aryloxy esters of AZT-monophosphate were inactive against HIV-2 in thymidine kinase deficient cells and thus are likely delivering the nucleoside (59). Anti-HIV nucleoside conjugates of ether and thioether phospholipids may effect a synergistic action of the lipid and the nucleoside in HIV-infected cells (60). Cyclic phosphates such as **20** have some activity against HCMV (about one-tenth that of GCV), possibly due to delivery of monophosphate (61).



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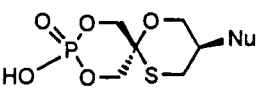


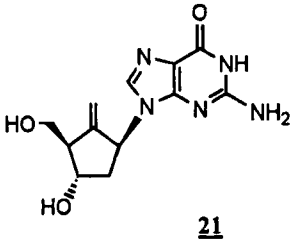
20

Carbocyclic Nucleosides - A recent review shows the remarkable diversity of carbocyclic nucleosides which has been provided by 30 years of creativity on the part of nature and organic chemists (62). Many drug candidates have emerged, but no drugs to date. The class deserves special mention this year because four members, all purines, are in various stages of clinical development for the treatment of viral infections: BMS-200475 (**21**, Phase 1), lobucavir (**22**, Phase 2), A-5021 (**23**, Phase 2), and 1592U89 (abacavir, **24**, Phase 3).

21, which is based on the template of carbocyclic 2'-deoxyguanosine (CDG), is a potent anti-HBV agent with an IC_{50} of 0.004 μM and a selectivity index of 8000 (63, 64). **22**, a cyclobutane analog of oxetanocin-G, has potent antiviral activity against herpesviruses (HSV, VZV, CMV) and is now being evaluated for the treatment of recurrent HSV infections and in HIV+ subjects coinfected with HCMV (65). Both **21** and **22** have shown efficacy in WHV-infected woodchucks and are now in clinical development for the treatment of chronic HBV infections (66).

Despite significant synthetic efforts, carbocyclic nucleosides containing cyclopropane have not been of interest until recently. The novel cyclopropyl analog **23** was reported to be more active than acyclovir or penciclovir in plaque reduction

assays against HSV-1 , HSV-2, HCMV, and VZV and more effective in mice with cutaneous HSV-1 infection. Clinical trials with 23 are in progress in Japan for the treatment of uncomplicated, acute, localized herpes zoster in immunocompetent patients (67).



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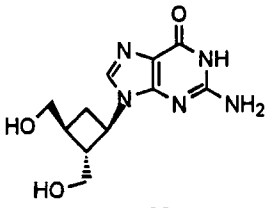

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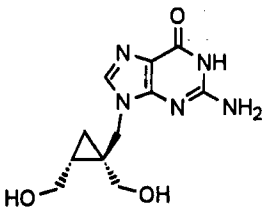

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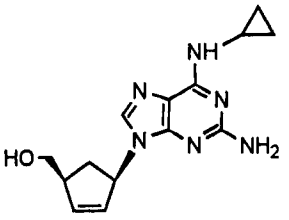
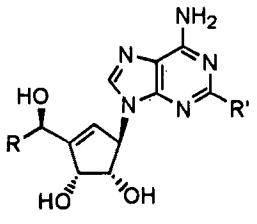


24

Presentations of pre-clinical research (68) and phase I/II clinical evaluations (69) of HIV reverse transcriptase inhibitor abacavir were not cited in previous reviews. Treatment of HIV-infected patients for 12 weeks with 24 resulted in sustained viral load reductions of >99%, unprecedented with nucleosides evaluated to date and comparable to results seen with protease inhibitors. Detailed summaries of the research that led to the selection of 24, including structure-activity relationships, resistance profile, and the unique mechanism of intracellular activation have appeared (70-72). The activation pathway, utilizing two new enzymes, provides a novel approach to selective nucleotide delivery and has been proposed to be responsible for the promising clinical results.



25 R = C≡CH, R' = NH_2

26 R = H, R' = F

Analogs of neplanocin A (NPA) were designed to avoid deamination by adenosine deaminase (73). The ethynyl analog 25 was 10-fold more potent than NPA against vaccinia virus and VSV. 2-Fluoro NPA. 26 was also resistant to adenosine deaminase and had antiviral activities comparable to or improved over NPA against viruses susceptible to S-adenosyl-homocysteine hydrolase inhibitors (74).

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Chapter 17. Non-Azole Antifungal Agents

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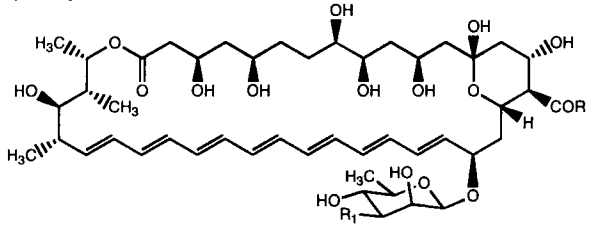
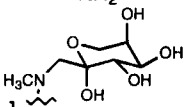
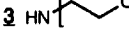
Introduction - The incidence of serious fungal infections continues to grow and is generally associated with host immunodeficiency. Current agents are inadequate in severely immune-compromised individuals and resistance is a growing problem (1,2). *Candida* spp., *Cryptococcus neoformans* and *Aspergillus* spp. are the most common causes of infection but there are a number of emerging pathogens (3). There is an urgent need for more effective and novel antifungal therapies.

Agents Affecting the Plasma Membrane - The exact nature of the interactions of amphotericin B (AmB, **1**) with sterols to form membrane channels has been under intense study. A spectroscopic investigation and molecular mechanics and dynamics simulations suggest that an aggregate coincides with channel activity (4,5). There are important intermolecular hydrogen bonding interactions among hydroxyl, amino and carboxyl groups that stabilize the channel in its open form and point to new directions for analog development (6,7). Heating solutions of **1** to 70° C forms a super-aggregated form that displays reduced red blood cell lysis and cytotoxicity with only a slight loss in antifungal activity (8).

Liposomal formulations are an important recent development in the area of drug delivery. The biopharmaceutical aspects of AmB formulations have been reviewed (9). ABLC, Ambisome® and ABCD have an improved therapeutic index relative to conventional AmB deoxycholate (10-12). AmB encapsulated in polyethylene glycol immunoliposomes showed higher lung concentrations compared to liposomal AmB that was not conjugated to a monoclonal antibody specific for murine pulmonary endothelia (13). Liposomal nystatin was as effective as nystatin in a neutropenic mouse model of disseminated *A. fumigatus* infection (14) but the liposomal structure is quickly lost when mixed with human plasma (15).



	R	R_1
1	OH	NH_2
2	OCH_3	
3		NH_2

Replacement of the polyene backbone of **1** with a biphenyl-diyne spacer of similar length resulted in a significant loss of activity (16). N-Methyl-N-D-fructopyranosyl AmB methyl ester (**2**) had an improved therapeutic index compared to **1** (17). A water soluble derivative, KY-62 (**3**), had similar potency to **1** and was well tolerated in mice (18). A semi-synthetic polyene, VB-28-3B methyl ester (**4**), showed activity similar to **1** and was 15-fold less toxic (19). Modification of patricin A (**5**) led to SPA-S-710 (**6**) and SPA-S-752 (**7**), derivatives with broad spectrum activity and an improved toxicity

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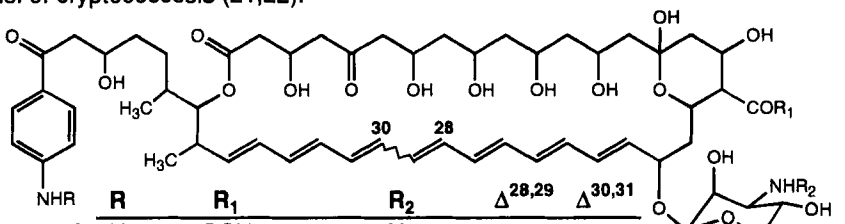
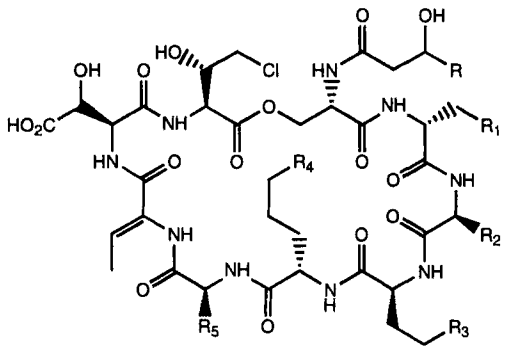
profile relative to 1 and 5 (20). Bis-amide 7 was more effective than 1 in a mouse model of cryptococcosis (21,22).

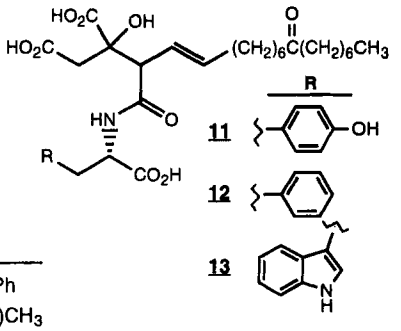


	R	R_1	R_2	$\Delta^{28,29}$	$\Delta^{30,31}$
4	H	OCH_3	H	trans	trans
5	CH_3	OH	H	cis	cis
6	CH_3	$NH(CH_2)_2NMe_2$	H	cis	cis
7	CH_3	$NH(CH_2)_2NMe_2$	Me_2NCH_2CO	cis	cis

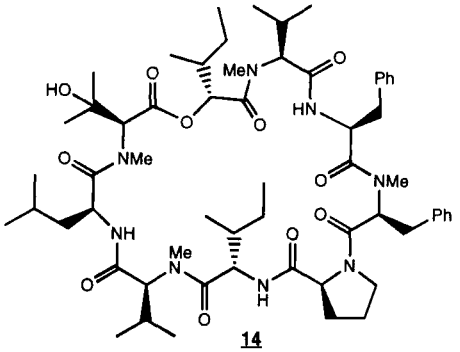
Syringomycin E (8), syringostatin A (9) and syringotoxin B (10) displayed broad spectrum fungicidal activity but lysed erythrocytes (23). Cholesterol increases the energy barrier for channel formation by 8 relative to ergosterol or stigmasterol (24). CAN-296, a 4.3 kDa carbohydrate consisting of 1,4-, 3,4- and 4,6-linked and terminal N-acetylglucosamine residues, was fungicidal to *Candida* spp. but was antagonized by Ca^{2+} (25). Poor activity was noted against *A. fumigatus* (26). The gene encoding histatin-3, a fungicidal peptide found in human saliva, was transferred to rodents via an adenovirus vector to produce salivary concentrations of over 1 mg/mL (27).



	R	R_1	R_2	R_3	R_4	R_5
8	C_9H_{19}	OH	$(CH_2)_2NH_2$	NH_2	$NHC(NH)NH_2$	CH_2Ph
9	$C_{11}H_{23}$	CH_2NH_2	$(CH_2)_2NH_2$	OH	NH_2	$CH(OH)CH_3$
10	$C_{11}H_{23}$	CH_2NH_2	H	OH	NH_2	$CH(OH)CH_3$



Viridiofungins A (11), B (12) and C (13) inhibit squalene synthetases at micromolar concentrations but exert their fungicidal effect by nanomolar inhibition of serine palmitoyltransferase (28). The target of aureobasidin A (14) has been identified as inositol phosphoceramide (IPC) synthase (29). The compound is fungicidal causing aberrant actin assembly which inhibits the normal budding process (30). Galbonolide A (15) also inhibits IPC synthase (31). The absolute stereochemistry of the galbonolides has been determined (32) and a total synthesis of galbonolide B (16) employed a Dieckmann condensation in the formation of the macrocycle (33). Khafrefungin (17) had an IC_{50} of 0.6 nM against IPC synthase from *C. albicans* (34).



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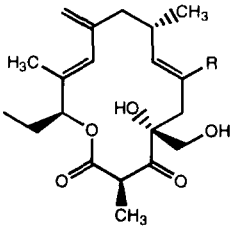
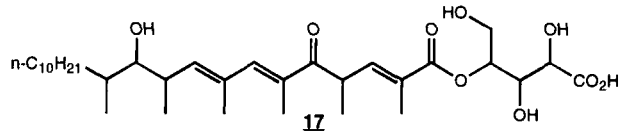


15 R = OCH_3

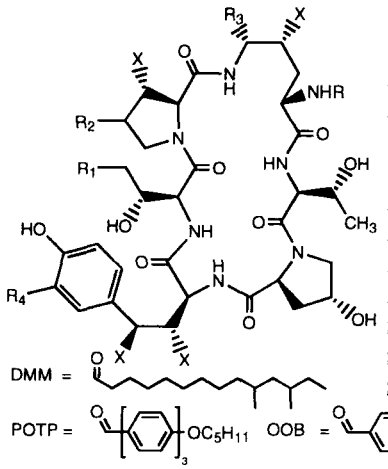
16 R = CH_3



17

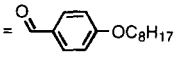
Inhibitors or Cell Wall Synthesis - The fungal cell wall is a complex structure and is essential due to a high intracellular osmotic pressure. The biosynthesis of carbohydrate cell wall polymers such as glucan and chitin is carefully orchestrated (35). Recent insights into the structure of the yeast cell wall and the function of enzymes involved in its biosynthesis suggest potential new antifungal targets (36-38). The gene encoding phosphatidylinositol 4-kinase was cloned and is important for cell wall integrity (39). β-N-Acetylhexosaminidase has been suggested as a cell wall target for the design of new therapeutic agents (40).

1,3-β-D-glucan synthase inhibitors encompass the lipopeptides, glycolipids and several miscellaneous compounds. New derivatives of pneumocandin B_0 (**18**) illustrate the importance of the homotyrosine phenol as a hydrogen bond donor in a series of *ortho*-substituted derivatives (41). A 3'-glycylamido derivative (**19**) was eight-fold more potent *in vivo* than **18**. The L-ornithine conjugate (**20**) displayed improved pharmacokinetics over L-733560 (**21**) but reduced antifungal potency (42). MK-0991 (**22**) (formerly L-743872) possessed potent fungicidal activity against fluconazole (FLU)-sensitive and resistant *Candida* spp., *Aspergillus* spp., *Histoplasma capsulatum*, and several clinically important molds (43-45). Compound **22**



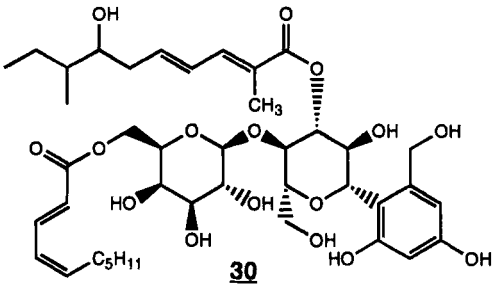
DMM =

POTP =

OOB = 

	R	R_1	R_2	R_3	R_4	X
18	DMM	$CONH_2$	H	OH	H	OH
19	DMM	$CONH_2$	H	OH	$NH(CO)CH_2NH_2$	OH
20	DMM	CH_2NH-Orn	H	$O(CH_2)_2NH_2$	H	OH
21	DMM	CH_2NH_2	H	$O(CH_2)_2NH_2$	H	OH
22	DMM	CH_2NH_2	H	$NH(CH_2)_2NH_2$	H	OH
23	POTP	H	α-CH_3	OH	H	OH
24	linoleoyl	H	α-CH_3	OH	H	OH
25	$COC_{15}H_{31}$	$CONH_2$	α-CH_3	OH	OSO_3H	OH
26	OOB	$CONH_2$	α-CH_3	OH	OSO_3H	OH
27	POTP	H	α-OH	H	H	H
28	POTP	H	β-NH_2	H	H	H
29	POTP	CH_2NH_2	β-NH_2	H	H	H

displayed potent activity in mouse models of infection with these pathogens (46-49) but poor activity against *C. neoformans*. Synergy was noted with FLU or AmB (50). Consistent inter-species pharmacokinetics and wide tissue distribution were seen (51). In a Phase II trial against *Candida* esophagitis, **22** demonstrated superior efficacy to AmB with fewer adverse reactions (52). LY303366 (**23**), a terphenyl side chain analog of echinocandin B (**24**), displayed potent activity against a broad range of clinical *Candida* isolates and *Blastomyces dermatitidis* but was slightly less potent against *C. parapsilosis* and inactive against *C. neoformans* (52-54). Compound **23** was fungicidal against FLU-resistant and -sensitive *Candida* isolates (56). The *in vitro* antifungal activity of **22**, **23** and FLU has been directly compared (57). Oral bioavailability of **23** in dogs was 9% (58). Phase I data for oral and iv formulations of **23** showed that it was well tolerated with a long half-life and was 3-5% orally bioavailable (59,60). N-alkylated side chain derivatives of **24** had good *in vitro* activity but lacked *in vivo* potency against *C. albicans* (61). FR901379 (**25**) and its octyloxybenzoyl side chain derivative, FR131535 (**26**), were active against *P. carinii* pneumonia (62). The synthetic cyclopeptamines, A-174591 (**27**), A-172013 (**28**) and A-175800 (**29**), were prepared by solid phase synthesis via macrocyclization on a resin (63). Substituents at R_2 showed that a β-amino group was optimal for overall activity. All three cyclic peptides had potent *in vitro* anti-*Candida* activity. Compounds **28** and **29** were active against *A. niger* and were one-tenth and one-fourth as potent as AmB in a *C. albicans* survival model (64). A new glycolipid, corynecandin (**30**), was a less potent 1,3-β-glucan synthase inhibitor than papulacandin B (65). *In vivo* expression of a complimentary mRNA of glucan synthase-1 resulted in reduced growth of *Neurospora crassa* (66). Derivatives of aurone (**31**) inhibited both glucan and chitin synthesis and were effective in a mouse model of candidiasis (67).



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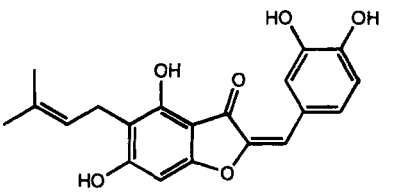
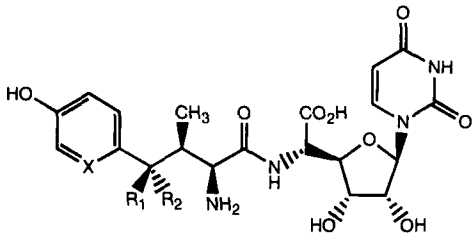


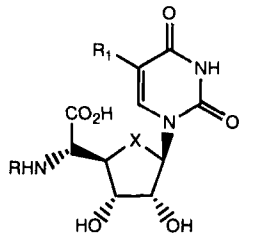
31

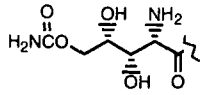
Nikkomycin Z (**32**) is a specific inhibitor of Chs2, one of three chitin synthase isozymes. It had good activity in a mouse model of pulmonary blastomycosis (68) but only modest activity against *H. capsulatum* (69). Single doses of up to 2 g were well tolerated in Phase I clinical trials although absorption was non-linear above 1 g (70). It was estimated that twice daily dosing of 250-500 mg would be sufficient to treat infections caused by *Coccidioidies immitis* and *B. dermatitidis*. The drug is degraded by plasma esterases from a number of species (71). The N-terminal aminoacid of nikkomycin B (**33**) was prepared via enzymatic resolution of an isoxazoline ester (72). A synthesis of the N-terminal aminoacid of nikkomycin Bz (**34**) has also been described (73). A palladium-mediated substitution reaction of an α-amino unsaturated lactone with bis-silylated uracil was utilized to construct (+)-carbocyclic uracil polyoxin

C (**35**) (74). A concise stereoselective synthesis of protected polyoxin C (**36**) was



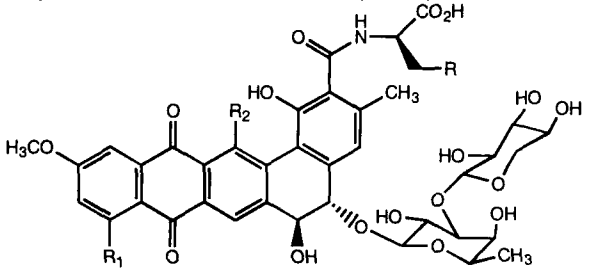
	X	R_1	R_2
32	N	H	OH
33	CH	OH	H
34	CH	H	OH



	R	R_1	X
35	H	H	CH_2
36	H	H	O
37		CH_3	O

accomplished through an oxabicyclo[3.2.1]octanone intermediate (75). A synthesis of polyoxin J (**37**) utilized the stereoselective addition of 2-lithiofuran (as a carboxylic acid equivalent) to a nitrone in the construction of the amino acid functionalities of the molecule (76). The carbohydrate portion of carbapolyoxins and carbanikkomycins was synthesized from norborn-5-en-2-yl acetate (77).

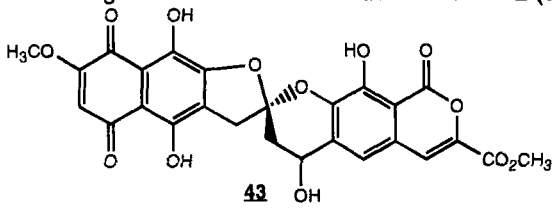
The pradamicins and benanomicins are naphthacenequinones that bind mannan in the presence of Ca^{2+} to disrupt the cell wall in pathogenic fungi (77-79). BMS 181184 (**38**) demonstrated good *in vitro* and *in vivo* activity against *Candida* and *C. neoformans* clinical isolates but weak activity against *C. parapsilosis* and *B. dermatitidis* (81). In Phase I studies, **38** elevated hepatic transaminases over a wide dose range (80). Benanomicin A (**39**) was fungicidal against a variety of organisms with MICs comparable to AmB and was intermediate in efficacy between AmB and FLU in mouse models of disseminated candidiasis, aspergillosis and cryptococcosis (82,83). 9-Deoxybenanomicin (**40**) had significantly reduced activity while O-methylated analogs **41** and **42** were inactive (84). Synthetic approaches to this class of compounds have been described (85-87).



	R	R_1	R_2
38	OH	OH	OH
39	H	OH	OH
40	H	H	OH
41	H	OH	OCH_3
42	H	OCH_3	OH

Protein Synthesis Inhibitors - Fungi are unique in that they require not only elongation factors 1 and 2 but a third factor (EF-3) that facilitates release of the deacylated tRNA from the E site. The function of EF-3 is fulfilled in higher eukaryotes by an intrinsic ATPase in 80S ribosomes (88). All of the elongation factor genes from *S. pombe* have been cloned (89). EF-3 from *S. cerevisiae* has been overexpressed and purified (90). The ribosome binding sites reside at the C-terminal end (91,92) and the two ATP binding motifs are indispensable for activity (93).

Purpuromycin (43) inhibits aminoacylation of tRNAs through a high affinity to all tRNA molecules (94). Aspirochlorine (44) selectively inhibits fungal protein synthesis but not through a direct interaction with EF-1 or EF-2 (95).



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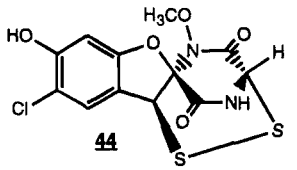
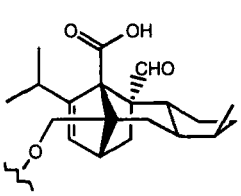


44

The mode of action of sordarin (45) is selective inhibition of elongation factor 2 (EF-2) in yeast (96,97). GM237354 (46), prepared via radical cyclization of a propargyl ether derived from a natural product (GM135402, 47), is a selective and potent inhibitor of the fungal enzyme (98,99). Moderate to excellent activity was seen against a broad range of sensitive and resistant organisms but 46 lacked activity against *C. krusei* (100). Compound 46 was highly effective in models of systemic and oral candidiasis, histoplasmosis, coccidioidomycosis and *P. carinii* pneumonia with low acute toxicity (101). No *in vitro* activity was seen against *A. fumigatus*, but high doses of 46 were modestly effective in a neutropenic mouse model of disseminated aspergillosis (102). The pharmacokinetics of 46 in rodents showed rapid clearance and a short half- life (103). Oral bioavailability in mice was 81%. Isobutyl ether derivative 48 was 1000-fold more active than 45 in a yeast inhibition assay (97).



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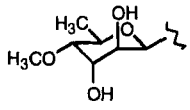

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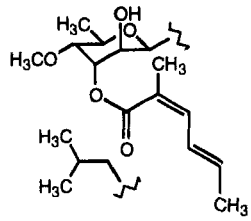

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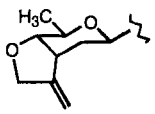
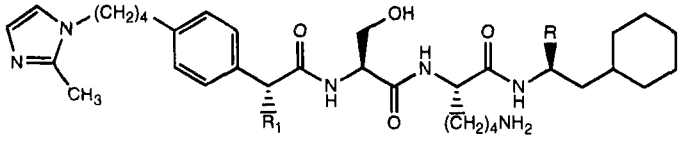


48

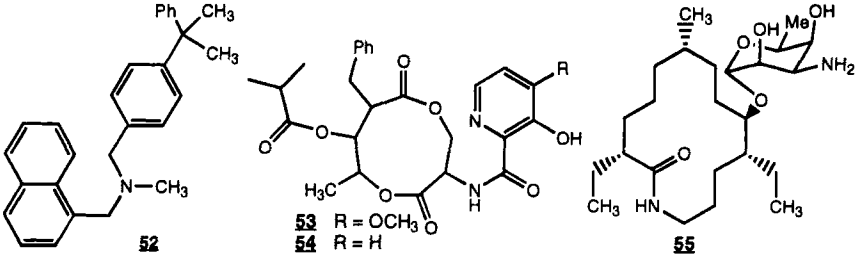
Miscellaneous Agents and Targets - An account of the development of fungal specific peptidomimetic N-myristoyltransferase (NMT) inhibitors has appeared (104). Conformationally constrained dipeptide analogs based on an octapeptide substrate were developed as inhibitors of *C. albicans* NMT (105). Further optimization led to 49, a 20 nM inhibitor with 400-fold selectivity over the human enzyme (106). Introduction of a carboxyl group (50) decreased activity 16-fold but increased fungal selectivity to over 2200-fold. Compound 51 had fungistatic activity against *C. albicans* at 51 μM.



	R	R_1
49	H	CH_3
50	CO_2H	CH_3
51	CO_2H	H

Myristic acid analog inhibitors of NMT, possessed modest, broad spectrum activity but were cytotoxic (107). SDZ SBA 586 (52), a non-competitive inhibitor of both fungal and mammalian squalene epoxidase, was more potent toward the *C. albicans* enzyme than terbinafine (108). Squalene epoxidase from *C. albicans* has been cloned (109). The synthesis and antifungal activity of (dl)-griseofulvin and substituted phenyl ring analogs was described (110). UK-2A (53) and UK-3A (54) showed broad spectrum

fungistatic activity and only weak cytoxicity (111). They inhibit mitochondrial respiration via the cytochrome bc_1 complex (112). FAS2 encodes fatty acid synthase



52 **53** R = OCH_3 **54** R = H **55**

in *C. albicans* and is essential for growth (113). The *C. albicans* topoisomerase II gene was isolated and shows only 40% homology to the human protein (114). It was shown that omeprazole inhibits plasma membrane H^+-ATPase from its extracellular face (115). An enantioselective synthesis of the antifungal agent SCH 38516 (**55**), employed a Mo-catalyzed macrocyclic ring closure metathesis reaction (116).

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SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

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Chapter 18. Blocking Interleukin-1 Action

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Introduction - Since the interleukin-1 field was last reviewed in this series (1), there have been some important advances in testing anti-IL-1 therapies in the clinic, in inhibiting the production of mature IL-1β and in understanding the signal transduction pathway that leads from the IL-1 receptor. The focus of this review will be to highlight those advances. For the basic biology surrounding the actions of IL-1 and the other members of the IL-1 pathway, the reader is referred to several recent reviews in the area (2-4).

Two IL-1 antagonists have entered into clinical trials, IL-1 receptor antagonist protein (IL-1ra) and the soluble type I IL-1 receptor. Data from these trials will be covered in this chapter. At the time of the previous review, caspase-1 (interleukin-1β converting enzyme or ICE) had been shown to be the enzyme responsible for processing pro-IL-1β into the mature, active cytokine. Since that time, a variety of inhibitiors of caspase-1 (both peptidic and nonpeptidic) have appeared in the literature. Lastly, there have been significant advances in understanding the events that occur intracellularly once IL-1 binds to the extracellular portion of the type-1 IL-1 receptor. This opens up the possibility of several new targets for the inhibition of IL-1 action. This chapter will review these advances for targeting the inhibition of IL-1.

INTERLEUKIN-1 RECEPTOR ANTAGONIST PROTEIN (IL-1RA)

IL-1ra binds to the type-I IL-1 receptor with high affinity and effectively competes with IL-1α or β. In order to block the biological activity of IL-1, however, high ratios of IL-1ra to IL-1 are required since binding of IL-1 to only 2-5% of the IL-1 receptors is required in order to elicit a signal. Clinical trials of IL-1ra have been conducted using the 17 kDa IL-1ra protein that was produced recombinantly in *E. coli.* This nonglycosylated form of IL-1ra was identical to the naturally-occurring glycosylated form except for the addition of an N-terminal methionine on the recombinant form of the molecule. The phase I trial of IL-1ra showed that it was safe and well tolerated (5). The positive outcome of this trial led to IL-1ra (also known as anakinra or Antril) being entered into a phase II trial for sepsis (6). This phase II trial was a randomized, placebo-controlled, open-label examination of IL-1ra for sepsis syndrome. The primary endpoint in this phase II study was the 28-day, all-cause mortality rate. Secondary end-points of the study included safety evaluation of IL-1ra, pharmacokinetics, effect on organ dysfunction, shock and cytokine synthesis (IL-1β, IL-6 and IL-1ra).

The results of this trial were particularly encouraging. The data indicated that IL-1ra had a dose-related effect in reducing the 28-day, all-cause mortality rate in patients with sepsis syndrome and septic shock. At the highest dose tested (133

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mg/h), there was a reduction in the mortality rate of 64%. Analysis of blood samples indicated that there was a dose-related increase in plasma IL-1ra levels at the end of the 72 hour infusion phase. These levels were at least 2 orders of magnitude greater than the IL-1ra levels found in the patients receiving placebo. In agreement with the Phase I data (5), IL-1ra was found to be safe and well-tolerated.

With the support of the data from the phase I and II trials, a large, multicenter phase III trial of IL-1ra was designed (7). A total of 893 patients received one of three treatments in this study, placebo, or IL-1ra at 1 mg/kg/h or at 2 mg/kg/h. The doses of IL-1ra used in this study were roughly equivalent to the highest two doses used in the phase II efficacy study. At the time of patient entry into the study, a predicted risk of mortality assessment was performed on each patient.

In this study, there was a trend toward reduction in all-cause mortality. Unlike the phase II study, however, this trend did not achieve statistical significance. Additionally, among patients that were in shock at study entry, there was no statistical difference in the mortality rate among any of the groups. There appeared to be a significant increase in survival time that was dose-dependent in those patients with organ dysfunction at study entry.

A second phase III trial of IL-1ra was designed to test the hypothesis that this compound would be efficacious in a high mortality risk group (8). This second phase III study sought to enroll a patient population of approximately 1300 people, which was deemed necessary to show statistical significance for the question being examined. The study design was purposely set to include patients who had organ dysfunction and/or shock at the time of their entry into the trial. The trial also included, however, patients that were less severely ill.

An interim analysis after nearly 700 patients had been enrolled in this phase III trial indicated that it was unlikely that IL-1ra would show efficacy in the more severely ill patient population that was targeted by this study. In all, 28 day mortality was assessed for a total of 906 patients in this study. An examination of the mortality rates for both the group as a whole as well as for the targeted group showed no significant differences in either case. While the group as a whole had a 9% difference in mortality rates between those receiving IL-1ra and those that did not, this difference was deemed to be not significant. In the group that had been targeted by this study, i.e., those that had the highest predicted mortality rate, the difference was even smaller, and again not significant.

The history of clinical trials in sepsis of IL-1ra point to a therapy that has marginal efficacy at best. Any findings of efficacy in one study, either performed perspectively or retrospectively, could not be repeated in subsequent studies. It must be concluded from these data that IL-1ra, in its present form, will not be efficacious for this indication.

These studies do conclusively show that this protein is safe and a significant immune response to it does not appear to be mounted, at least out to the 28 day endpoint of these clinical trials. Based on these data, a trial of IL-1ra in patients with active rheumatoid arthritis has been carried out (9). The total length of the study was seven weeks, with multiple doses of IL-1ra being administered via subcutaneous injection. The results showed that IL-1ra was safe and well-tolerated, but the number of patients enrolled in the trial was too small to detect a significant difference favoring treatment. It appeared that daily dosing was more effective than weekly dosing, and

this would agree with the reported pharmacokinetics of IL-1ra. A larger, placebo controlled trial is necessary to definitively say whether or not IL-1ra will be efficacious for this indication and also if it would be a cost-effective treatment.

SOLUBLE TYPE I IL-1 RECEPTOR

There are two forms of the IL-1 receptor, type I and type II (4). Both receptors bind IL-1, but only the type I receptor is responsible for mediating a signal. The type II receptor is thought to be a decoy receptor whereby a soluble version of this receptor is shed from the cell membrane naturally and acts to negatively regulate the actions of IL-1. This receptor has a higher affinity for IL-1 than it does for IL-1ra, and therefore could function as an additional modulator of IL-1 action in vivo. A soluble version of the type I receptor has been made recombinantly and overexpressed in mammalian cells. This protein has been entered into clinical trials with phase I/II trials having been performed in rheumatoid arthritis and the late-phase allergic response. Data from those two limited trials will be briefly reviewed here.

The first trial of sIL1RI was performed to study modification of the late-phase allergic response in healthy volunteers (10). This phase I/II trial also studied the safety of the protein when delivered subcutaneously. An allergen was injected intradermally on each subjects forearm followed by subcutaneous injections of sIL-1rI at the same site. Allergen and placebo were injected into the contralateral arm of each subject, therefore, each subject acted as the placebo control as well.

The data indicated that at the lowest two doses of sIL1rI, there was a significant reduction in the late-phase response in the arms receiving the drug in comparison to the contralateral arms. At the three highest concentrations of sIL1rI, a decrease in the LPR at both the arm that received the drug as well as the placebo arm was observed. This indicated that sIL1rI was having a systemic effect. Finally, there did not seem to be any toxicity associated with any of the dosage levels that had been administered in this study. The caveat with this study, though, is that it was a very short-term study with a single injection of sIL-1rI.

The second trial was longer term (28 days) and examined the effect of sIL-1rI in patients with active RA (11). The drug was delivered once each day for 28 days and the route of administration was again subcutaneous. Physical examinations and laboratory assessments were made at various times out to 57 days after the start of the study. The results of this study were less encouraging than the first study. Only one of eight patients demonstrated any clinically meaningful improvement in symptoms, and this was at the highest dose of sIL-1rI tested. That the doses of sIL-1rI used were functional was indicated by a reduction in the levels of IL-1α observed.

Both of these studies were conducted with a very limited number of patients, so the results must not be over-interpreted. These studies indicate that further examination of rhu sIL-1rI may be warranted. However, in light of the greater affinity of the type I receptor for IL-1ra than the type II receptor, trials of the soluble type II IL-1r may have a stronger possibility of showing efficacy. If trials of the sIL-1rII are underway, no data have yet been presented on the results.

CASPASE-1

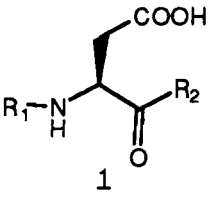
Caspase-1 is a cysteine protease that converts the inactive proform of IL-1β to the active inflammatory cytokine and hence represents an attractive target for the modulation of the effects of IL-1β (12,13). The therapeutic value of caspase-1 inhibitors may also be enhanced by indirect effects of caspase-1 inhibition on levels of IL-1α (14) as suggested by data from caspase-1 knockout mice. Studies in caspase-1 deficient mice have also pointed to possible effects of caspase-1 inhibition on interferon-γ (INF-γ) (15,16). This effect may be accounted for by the fact that interferon-γ inducing factor (IGIF; IL-18) can also be activated by caspase-1. Interest in caspase-1 has expanded with the identification of an entire family of related human caspases, of which caspase-1 is the first example. Members of the caspase family have been implicated in the process of apoptosis (17,18). However, focusing only on the issue of caspase-1 inhibitors for IL-1 modulation, the specificity of caspase-1 inhibitors vs. the other caspases still emerges as an important consideration.

Peptidic Inhibitors -- Since the subject was last reviewed in this series (1), a variety of peptidic inhibitors of caspase-1 have been reported. These compounds are largely based on the reported substrate specificity of caspase-1 for Ac-Tyr-Val-Ala-Asp at S4 through S1. Another common feature of these inhibitors is the presence an electrophilic group capable of reacting with the active site cysteine either reversibly or irreversibly. As shown in Figure 1, a variety of structural types have been reported, including aldehydes (19,20), acyloxymethylketones (21-23), aryloxymethylketones (24), heteroaryloxymethylketones (25,26), ketones (27) and activated ketones (28,29). These compounds provided an important initial understanding of the SAR of caspase-1 inhibitors. A substantial portion of this early peptide work has been comprehensively reviewed (13,30).

Although not likely drug candidates, these peptidic compounds have proven useful in initial studies of the effects of caspase-1 inhibitors *in vivo*. Work with these compounds has nicely supplemented proof of concept work in caspase-1 deficient mice, which also support the potential therapeutic value of caspase-1 inhibitors (14,31-35). A peptidic aldehyde (Ac-Tyr-Val-Ala-Asp-H) and two acyloxymethyl-ketones, (Z-Val-Ala-Asp-dichlorobenzoyloxymethylketone and its ethyl ester) have been shown to suppress the induction of IL-1β following LPS or zymosan stimulation in mice (36-38). Furthermore, the ethyl ester of Z-Val-Ala-Asp-dichlorobenzoyloxy-methylketone dosed IP has been reported to delay onset of symptoms and to reduce disease severity in collagen induce arthritis in mice (37). This same compound was shown to reduce inflammation when dosed PO in carrageenin induced paw edema in rats (39). It also decreased severity of disease and lethality in a model of pancreatitis in rats (40). Mice dosed with Z-Val-Ala-Asp-fluoromethylketone were protected from apoptotic liver damage (41,42). In studies relevant to potential stroke indications, peptidic fluoromethylketones and acyloxymethylketones dosed ICV have been shown to reduce ischemic brain damage following middle cerebral artery occlusion in rats (43,44).

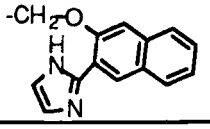
Peptidomimetics -- Recently, peptidomimetic inhibitors of caspase-1 have been disclosed in the literature. The design of these inhibitors was based on key SAR information gleaned from peptidic caspase-1 inhibitors, including the importance of a P1 Asp. Also considered was the β-sheet type hydrogen bonding pattern the peptidic inhibitors form with the enzyme. With these considerations in mind, the peptidomimetic inhibitors were designed retaining the important P1 Asp, but with the P3-P2 residues

Figure 1



1

Reversible Inhibitors

R_1	R_2	Ki nM	Ref.
Ac-Tyr-Val-Ala-	-H	0.76	(19)
Ac-Tyr-Val-Ala-	$-CH_5Ph$	18.5	(27)
$PhCH_2CH_2(CO)$Val-Ala-	$-CH_2S(CH_2)_3Ph$	11	(27)
alloc-	$-CH_2S(CH_2)_3Ph$	27000	(28)
alloc-		90	(29)

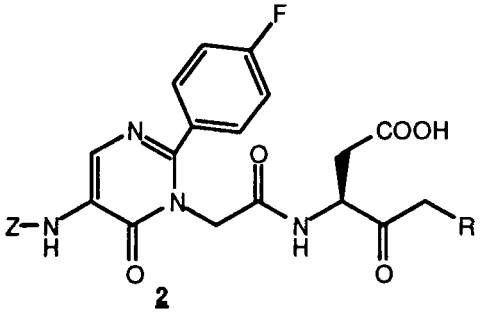
Irreversible Inhibitors

R_1	R_2	Kobs/[I] ($M^{-1}S^{-1}$)	Ref.
Z-Val-Ala-	$-CH_2DCB$	407,000	(13)
Z-Val-Ala-	$-CH_2PTP$	280,000	(13)
Z-Val-Ala-	$-CH_2DPP$	117,000	(13)

DCB = (2,6-dichlorobenzoyloxy: PTP = (1-phenyl-3(trifluoromethyl)pyrazol-5-yl)oxy; DPP = (diphenylphosphinyl)oxy

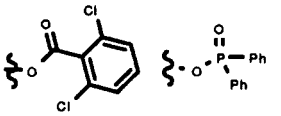
replaced by a dipeptidemimetic having the desired hydrogen bonding pattern. In addition, x-ray crystal structures of caspase-1 with Ac-Tyr-Val-Ala-Asp based inhibitors bound at the active site have been reported and have aided the design of peptidomimetic inhibitors (45,46).

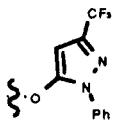
In three related series of caspase-1 inhibitors, the P3-P2 residues have been successfully replaced with a pyridone or pyrimidone-based dipeptidemimetic. Irreversible inhibitors (2) were only 2- to 3-fold less potent than the corresponding Z-Val-Ala-Asp peptidic inhibitors as measured by their second order rate constants



2

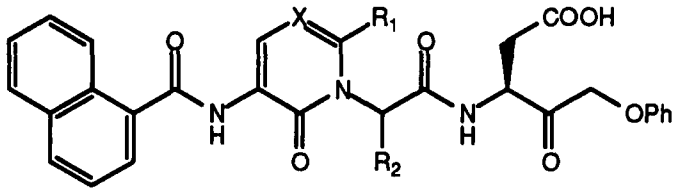
R =





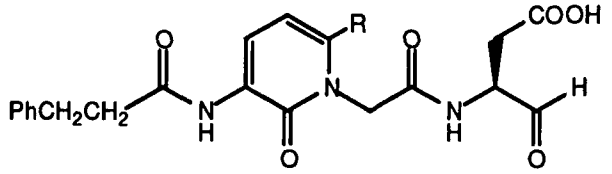
(47). Replacement of the p-F-phenyl had only minimal effect on activity, suggesting that this group was not significantly contributing to binding.

A related series of reversible phenyloxymethylketones **3**-**6** provide further SAR information (48). In this series, an N-terminal 2- or 1-naphthoyl gave significantly better potency than the corresponding benzyloxycarbonyl. R1 = n-butyl was best, however, it was only 2-fold more potent than the corresponding unsubstituted derivative. R2 substituents significantly increased activity with ethyl being most potent. Finally, it was found that pyridone was more potent than the corresponding pyrimidone.



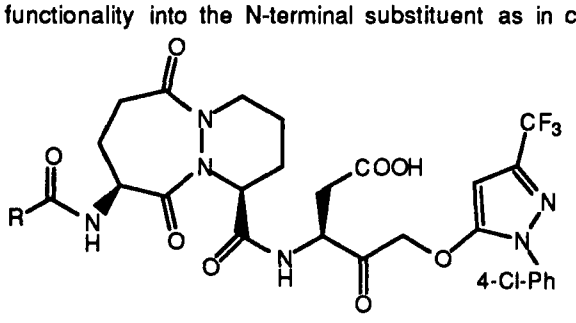
3 X = N R_1 = nBu R_2 = H
4 X = CH R_1 = H R_2 = H
5 X = CH R_1 = H R_2 = Et
6 X = CH R_1 = nBu R_2 = H

In a related series, the use of x-ray crystallography and molecular modeling led to improvements in binding at R_1 (49). The benzyl analog **7** was designed based on an x-ray crystal structure of a pyridone inhibitor bound to the active site of caspase-1 and was found to be 32-fold more potent than the corresponding unsubstituted compound **8**. X-ray crystallography of the corresponding irreversible dichlorobenzoyloxymethyl-ketone confirmed that the benzyl binds in a hydrophobic region near the P2 pocket.

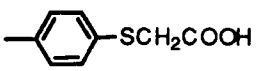


7 R = CH_2Ph
8 R = H

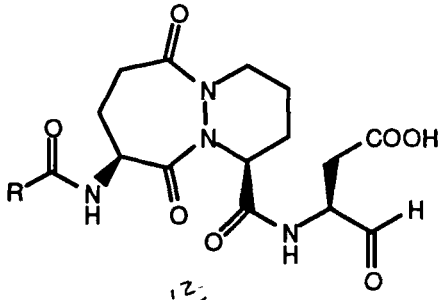
A study replacing the P3-P2 residues with a variety of constrained dipeptide mimetics identified pyridazinodiazepines as highly potent inhibitors of caspase-1 (50). Irreversible inhibitor **9** was comparable in activity to the corresponding Z-Val-Ala-Asp peptidic inhibitor. Further improvement in activity was obtained by incorporating acidic functionality into the N-terminal substituent as in compound **10**. Similarly for the



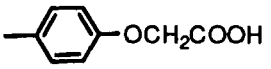
9 R = $-OCH_2Ph$

10 R = 

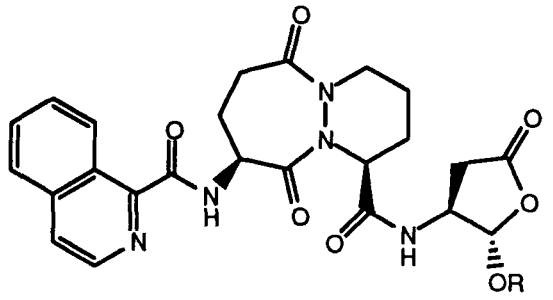
corresponding reversible carboxaldehyde inhibitors, pyridazinodiazepine **11** was comparable in potency to the corresponding Z-tripeptide aldehyde Z-Val-Ala-Asp-H. As with the irreversible series, activity was further increased by the addition of an acidic functionality on the N-terminal substituent.



11 R = Ph

12 R = 

Compound **12** showed interesting activity *in vivo*, suppressing IL-1β induction more than 95% in mice dosed at 100 mg/kg IP. Furthermore, it is reported to have 12-16% oral bioavailability in dogs with a clearance rate of ca. 7 mL/min/kg (50). Additional SAR of the pyridazinodiazepines has appeared in the patent literature (51), including cyclic prodrugs **13**-**14**, which exhibit 32-35% bioavailability, in rats and show oral activity in a collagen induced model of arthritis.



13 R = -Et
14 R = -cyclopentyl

In summary, the field of caspase-1 inhibitors has moved forward significantly in the past year with the disclosure of potent, nonpeptidic inhibitors. And substantial current interest remains in the concept of caspase-1 inhibition as a means of modulating IL-1β production.

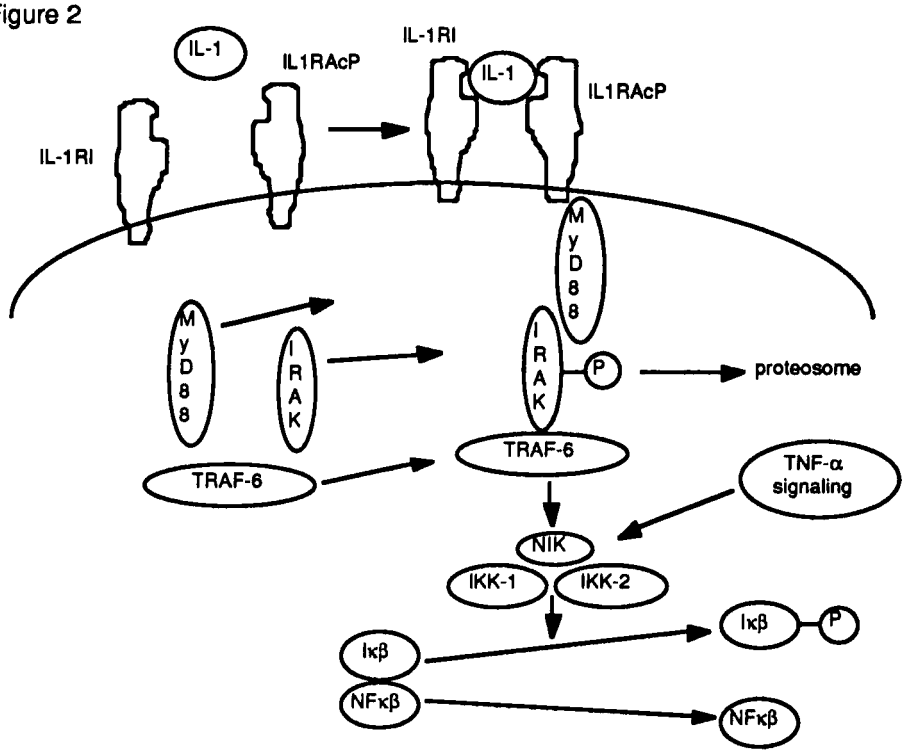
INTERLEUKIN-1 SIGNAL TRANSDUCTION

At the time of the last review of IL-1 in this series (1), little was known about the events that occur intracellularly after the cytokine binds to its receptor. Since then, however, there has been a wealth of new knowledge about these events that has begun to fill in the picture of the individual steps in the IL-1 signal transduction pathway.

There appear to be at least four proteins that are involved at the start of the IL-1 signaling cascade, in addition to IL-1 itself (see Figure 2). The first is the IL-1 type I receptor, which has a long cytoplasmic tail that lacks any intrinsic catalytic activity (4). The second protein is also a transmembrane receptor molecule called the IL-1 receptor accessory protein (52). The third protein involved in this complex (and the first intracellular one) is MyD88, which then binds to IL-1 receptor associated kinase or IRAK (53). IRAK was shown to be specific for signaling via either IL-1 or IL-18 (54,55).

Activation of IRAK was shown to be rapid, occurring within 30 seconds after binding of IL-1 to the receptor (56). The activation of this kinase was transient and it appears that downregulation of its activity was accomplished by degradation of IRAK by proteosomes (57).

Figure 2



A rough outline of events for the IL-1 signal transduction cascade can now be described (2). The first step would be binding of IL-1 to the type I IL-1R followed by recruitment of IL-1AcP to form a trimeric extracellular complex. IRAK is then rapidly translocated from the cytoplasm to the intracellular side of this complex where it binds to MyD88 and becomes autophosphoryated. Within a few minutes of its association with the receptor complex, IRAK is degraded by proteosomes.

The pathways for IL-1 and TNF-α signaling converge at NFκβ-inducing kinase (NIK) (58,59). This kinase activates the Iκβ kinase signalsome (IKK-1 and IKK-2) (60,61), which directly phophorylates Iκβ (62) and targets Iκβ for degradation. NFκβ is then translocated to the nucleus where it activates transcription. This sequence of events that follow IL-1 binding to its receptor opens up the possibility of three new targets for the inhibition of IL-1 signaling. The first possibility is IRAK, which functions only in IL-1 signaling. Two other possible targets, however, could function to block both TNF-α and IL-1 signaling. These are the IKK signalsome and NIK. Further work will be required to determine if any of these kinases are viable targets to block IL-1 signaling.

Conclusions - Considerable interest remains for inhibiting the actions of IL-1 with multiple targets both inside and outside the cell being explored. The first strategies to make it into the clinic were slight modifications of naturally-occurring proteins (IL-1ra

and sIL-1rI). While there have been some positive clinical results at times with these agents, neither has proven to be efficacious against the targeted diseases.

There are several targets where development of small molecule inhibitors may succeed at inhibiting the actions of IL-1. Caspase-1 inhibition would prevent the initial production of mature IL-1. A number of peptidic and non-peptidic inhibitors have been described, and several of these have been tested in animal modles of disease. Data are available that indicate some of these compounds have oral bioavailability and the required potency that may make them viable development candidates. The elucidation of the signal transduction events that occur after IL-1 binds to its receptor has opened up three possible new targets that may make viable targets for inhibiting the action of IL-1.

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Chapter 19. β_3 Adrenergic Receptor Agonists for the Treatment of Obesity

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Introduction - Since the identification of a third β adrenergic receptor (β3 AR) over ten years ago (1), there has been intense interest in developing selective β3 AR agonists for the treatment of obesity and type 2 diabetes. Early compounds, which were identified based on their ability to stimulate lipolysis in rat brown adipose tissue (BAT) in the absence of effects on heart rate (a β1 effect) and tracheal relaxation (a β2 effect), failed in the clinic. With the cloning of the human receptor (2), and the subsequent determination that the pharmacology of the rat and human receptors were distinct (3), there has been renewed effort to identify compounds which are selective for the human receptor. This review will thus focus on advances in the identification of human β3 AR agonists, as well as developments in β3 adrenergic receptor biology since this topic was last reviewed in this series (4). Several other reviews have recently appeared (5–,6,7,8).

RECENT DEVELOPMENTS IN β3 ADRENERGIC RECEPTOR BIOLOGY

Evidence for the Existence of a Functional β3 AR in Humans - While it is clear that the β3 AR plays a key role in mediating thermogenesis in rodents, and that specific β3 AR agonists increase metabolic rate and lead to weight loss in obese rodents, the role of β3 ARs in humans remains controversial. In human newborn perirenal brown adipose tissue (BAT), the levels of β1, β2, and β3 mRNA were found to be 28, 63, 9%, respectively, of the total adrenergic receptor mRNA; however, in adult human abdominal white adipose tissue (WAT), no β3 mRNA was detected by Northern blot analysis (9). In a separate study (10), using a sensitive and specific RNase protection assay without previous PCR amplification, β3 mRNA was detected in human WAT, gall bladder, and small intestine, confirming earlier reports. It was also found to a lesser extent in stomach and prostate, but was absent in cerebral cortex, cerebellum, liver, pancreas, gastrocnemius and soleus muscle, left ventricle, lung, corpus cavernosa and kidney.

CGP 12177A (1), a mixed β1/β2 antagonist with partial agonist activity at the human β3 AR (11), has been used extensively to determine whether stimulation of β3 AR in human fat induces lipolysis. In a large study of WAT from 50 patients, lipolysis induced by 1 was found to be more pronounced in isolated fat cells from abdominal than from subcutaneous WAT (41% vs. 31% intrinsic activity, respectively) (12). The non-selective β AR antagonist bupranolol was better able to block this effect than either the β1 selective CGP 20712A or the β2 selective ICI 118,551 (pA_2 = 7.17, 6.26, 6.05, respectively). Discrepancies seen in the literature could be explained in part by the large interindividual differences seen in the lipolytic response to 1, with lipolysis correlating with norepinephrine sensitivity and responsiveness to other lipolytic agents acting via cAMP. In addition, the lipolytic response was lower when tissue fragments were used instead of isolated fat cell suspension, and the sensitivity of the lipolysis assay itself may be a factor. In a separate study (13), the lipolytic effect of 1 in human subcutaneous WAT was not blocked by the newly described β3 AR antagonist, SR 59,230A (vide supra); however, the activity of this compound at the human receptor has not been reported.

Using microdialysis, 1 was shown to stimulate lipolysis, as measured by increased glycerol release, in intact subcutaneous adipose tissue (14). Pretreatment with a dose of propranolol which blocked glycerol release by dobutamine, a β1 AR agonist, and terbutaline, a β2 AR agonist, had no effect on glycerol release by 1. An increase in

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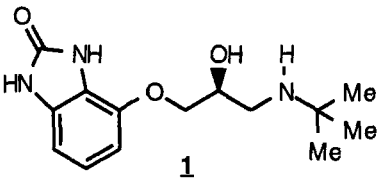
nutritive blood flow induced by 1 was also evident in a separate dialysis study, indicating the possible presence of β3 ARs in the vasculature of subcutaneous WAT (15).



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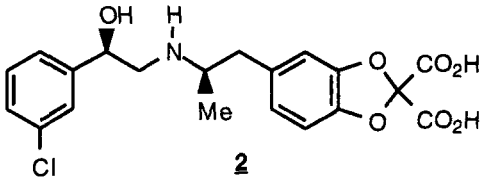
β3 AR Polymorphism - A Trp64Arg mutation in the β3 AR gene was first described in the Pima Indians, a population with a high incidence of obesity (16). While not found to be more prevalent among obese subjects than non-obese, the mutation was associated with an earlier onset of type 2 diabetes mellitus and a lower resting metabolic rate in obese homozygous individuals. A large number of studies, recently reviewed (17), suggest additional associations between the Trp64Arg β3 AR and hyperinsulinemia and insulin resistance, higher body mass index, an increased capacity to gain weight, resistance to weight loss, increased blood pressure, and coronary heart disease. This remains an area of intense investigation, and there is some debate as to the significance of the mutation in obesity and type 2 diabetes, as a number of studies have shown no linkage (17,18).

In the human receptor, the substituted amino acid at position 64 lies at the junction of the first transmembrane spanning domain and the first intracellular loop. Of the nine known species homologues, only the human and the guinea pig (which contains a Cys residue) do not have Arg at this position (19). The mutant receptor has been cloned and expressed in CHO cells, and was found to be pharmacologically identical to wild type with respect to agonist binding affinity and stimulation of cAMP (20). In addition, the two receptors showed similar rates of desensitization in response to isoproterenol exposure. A second study, in which the receptors were expressed in CHO-K1 and HEK293 cells, confirmed that agonist affinity was identical, but found that cAMP accumulation in both cell lines was lower in cells expressing the mutant receptor than in those expressing the wild type (21). This reduction did not appear to be due to enhanced interaction of the Trp64Arg β3 AR with the G-protein Gi, since pertussis toxin treatment did not restore activation, and thus could possibly be due to decreased coupling efficiency with Gs.

Using microdialysis, *in vivo* lipolysis in subcutaneous adipose tissue of Pima Indians homozygous for either the Arg or Trp allele was assessed (22). No differences were seen in isoproterenol stimulated lipolysis; however, in subcutaneous WAT, the β3 AR component of total lipolysis may be small. When lipolysis in isolated visceral fat cells from Caucasians either homozygous for Trp64 or heterozygous for Trp64Arg was compared, no differences were seen with both 1 and norepinephrine stimuation (23).

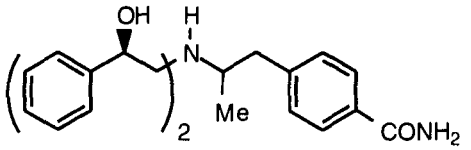
Regulation of Uncoupling Proteins by the β3 AR - The β3 AR is believed to exert its metabolic effects through activation and upregulation of the brown fat specific mitrochondrial uncoupling protein, UCP, now designated UCP1. In obese yellow KK mice, chronic treatment with the rodent selective β3 agonist CL 316,243 (BTA-243, 2) led to increases in UCP1 mRNA in BAT, WAT, and surprisingly, also in gastrocnemius and quadricep muscles (24). Protein was detected in skeletal muscle by Western blot, and this was localized to the mycocyte mitochondria using gold labeling. No UCP1 was detected in the skeletal muscle of control non-obese C57BL mice treated with 2. Since no β3 AR was detected in muscle, this may be an indirect effect, but leaves open the possibility that ectopic expression of UCP1 may contribute to the effectiveness of β3 agonists in increasing metabolic rate.

Recently two additional uncoupling proteins have been identified in adipose tissue. UCP2, which is 59% homologous to UCP1, is ubiquitously expressed in humans, with



2

mRNA detected in adipose tissue, skeletal muscle, lung, heart, placenta and kidney. High levels were also found in spleen, thymus, leukocytes, macrophage, bone marrow and stomach, with low levels in brain and liver (25,26). Regulation of UCP2 appears to be different from that of UCP1. Cold exposure or treatment with **2** did not alter mRNA levels of UCP2 in BAT, WAT, thigh muscle and liver of male Swiss mice; however, UCP2 levels were dramatically increased in WAT of A/J and C57BL/6J mice when fed a high fat diet (25). In contrast, in rats UCP2 mRNA was reportedly increased in BAT, heart and soleus (but not tibialis anterior and gastrocnemius) muscle in response to cold exposure, and in BAT following treatment with Ro 16-8714 (**3**), a β3 AR agonist (27). This effect on BAT was not seen in rats following treatment with β3 agonist **2**, though a slight increase in UCP2 levels in WAT was observed (28).



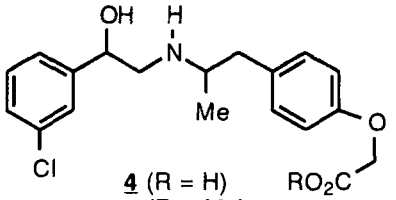
3

In contrast to UCP2, UCP3 is expressed primarily in skeletal muscle in humans, with lower amounts in heart, thyroid, and bone marrow, and little or none in WAT (28–30). In mice (29) and rats (28,30), UCP3 levels are high in skeletal muscle and BAT, with lower amounts in WAT and other tissues. Following treatment of rats with **2**, UCP3 is markedly upregulated in WAT, suggesting that it may contribute to β3 AR agonist-induced thermogenesis (28). Both UCP2 and UCP3 expression is upregulated in muscle during starvation, thus they may be important for metabolic adaptation to food deprivation (27,28,31).

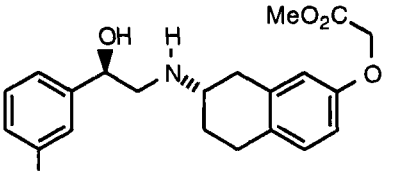
Regulation of Leptin by the β3 AR - The β3 AR appears to play a key role in the action and regulation of leptin (32). This hormone, the product of the *ob* gene (33), is secreted by adipocytes and, acting via the hypothalamous, inhibits food intake and stimulates metabolic rate (34). Leptin-induced activation of the sympathetic nervous system and the resultant β3 AR-mediated thermogenesis in BAT may be responsible for the latter effect (35).

In isolated rat white adipocytes, **2** causes a concentration-dependent decrease in insulin-stimulated leptin release (36), indicative of a role for the β3 AR in a negative feedback loop. These results have been confirmed in mouse brown adipocytes differentiated in culture. In this system, β3 AR agonist BRL 37344 (**4**) (the active metabolite of prodrug BRL 35135, **5**) decreases leptin secretion and inhibits *ob* gene expression with an EC_{50} of 0.3 nM, apparently by destabilization of *ob* gene mRNA (37). Treatment of mice with **2** (38) or **5** (39) causes a decrease in both circulating leptin levels and *ob* gene expression in WAT. A decrease in leptin levels generally results in increased food intake, but in animals treated with **2**, an acute decrease in food intake was seen. No effect of **2** on leptin levels or feeding was seen in β3 AR knock-out mice (37). A similar reduction in food intake and *ob* gene expression in WAT was noted in re-fed rats treated with **1** (40). In a separate study (41), β3 AR knock-out mice fed a high fat diet showed greater increases in percent body fat than control mice; however, no differences in leptin levels were observed. Thus, while β3 AR activation leads to leptin downregulation, β3 AR deactivation, which causes an increase in fat deposition, does not necessarily lead to leptin upregulation.

Cardiac β3 and Putative β4 ARs - In human ventricular preparations, concentration-dependent negative inotropic effects were seen with **4**, SR 58611A (**6**), **2**, and, to a lesser extent, **1** (42). Pretreatment with β1 antagonist metoprolol or β1/β2 antagonist nadolol did not alter the effects due to **4** whereas pretreatment with the non-selective antagonist bupranolol shifted the dose-response curve to the right. The response to **4** was diminished by pertussis toxin pretreatment, indicating the possible involvement of Gi coupling in this effect (42). In contrast to a previous study (10), expression of the β3 AR was detected in human ventricle by RT-PCR (42). No mRNA from hormone sensitive lipase was detected in the preparations, indicating that fat contamination was not the source of the β3 AR transcripts. This potential for decreased contractility may be problematic in patients with chronic heart failure (43). Negative inotropic responses to β3 AR agonists were not confirmed in a limited study of right ventricular trabeculae from three patients (44). In particular, **1** caused a concentration-dependent increase in cardiac stimulation (44).



4 (R = H)
5 (R = Me)



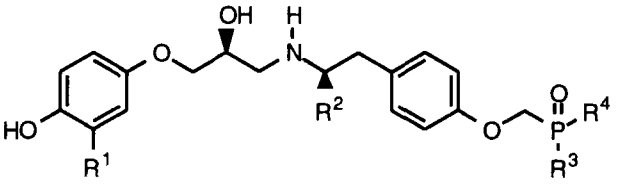
6

The existence of a cardiac β AR distinct from the β3 AR, the putative β4 AR, has been proposed (45,46). Its existence is based primarily on the cardiostimulatory effects of classic β AR antagonists such as **1**, which pharmacology is distinct from that of their β3 AR mediated effects. There is some debate as to the existence of a fourth AR, especially in light of the fact that the pharmacology of β3 AR agonists varies, for example, with cloned receptors in membranes vs. whole cells (47).

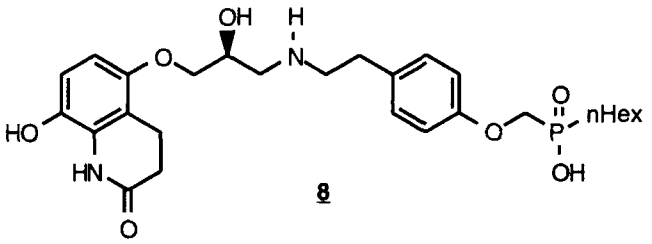
DEVELOPMENT OF HUMAN β3 AR AGONISTS

Cloned Receptor Assays for the Identification of Agonists - With the discovery of species differences in the potency and selectivity of β3 AR agonists, researchers have increasingly relied on the use of cloned human receptor assays for the identification of human selective compounds (48). The affinity and efficacy of β3 AR agonists in a cloned receptor assay is dependent upon a number of factors including receptor density and the nature of the assay, i.e., cAMP accumulation in whole cells vs. adenylyl cyclase activation in membrane preparations (49). Agonist **1** had an EC_{50} of 1113 nM and an instrinsic activity (IA) of 0.56 in a cell membrane assay with a receptor density of 130 fmol/mg and an EC_{50} of 263 nM (IA 0.87) in one with with a receptor density of 3000 fmol/mg. In intact cells, its EC_{50} was 260 nM (IA 0.45) in the former clone and 70 nM (IA 0.87) in the latter (49). These differences should be considered when making comparisons among compounds assayed in different laboratories.

Aryloxypropanolamines - There are two main classes of compounds which are known to bind with high affinity to β ARs, the aryloxypropanolamines and the arylethanol-amines. Interest in the former class of compounds, which are typically beta blockers, stems from the fact that aryloxypropanolamine **1**, a β1/β2 AR antagonist, has partial agonist activity at the human β3 AR (11). Using a "simplified template approach" in which the left hand side of the aryloxypropanolamine was optimized for potency and intrinsic activity and the right hand side for potency and selectivity, phosphinic acids **7** (β3 EC_{50} = 120 nM, IA 1.29)and **8** (EC_{50} = 0.29 nM, IA 0.84) were identified (50). The related derivative **9** is reportedly highly selective with a β3 EC50 of 1.7 μM, (IA 0.70) and β1 and β2 Ki's of 288 and 269 μM, respectively (51). Phosphonate derivatives **10** and **11** have increased potency (EC_{50} = 10 nM for both), though reduced selectivity (β1 and β2 Ki's = 130 and 120 nM, respectively for **11**) (52).

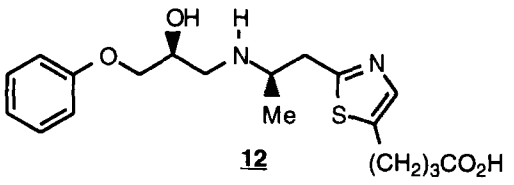


	R^1	R^2	R^3	R^4
7	$NHSO_2Me$	Me	OH	Ph
9	H	H	OH	cHex
10	$NHSO_2Me$	Me	$(CH_2)_3Ph$	$(CH_2)_3Ph$
11	H	Me	Ph	Ph



8

A series of thiazolylalkylacids was explored. The propionic acid BMS-187257 (12, β3 EC_{50} = 630 nM, IA 0.72) was optimal, though only 2 to 3-fold selective over β1 and β2 (53). Starting from an arylethanolamine lead, and using combinatorial chemistry as an optimization tool, amide 13 was identified as a potent and selective β3 AR agonist (54).



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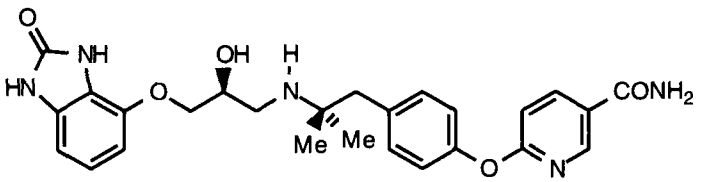
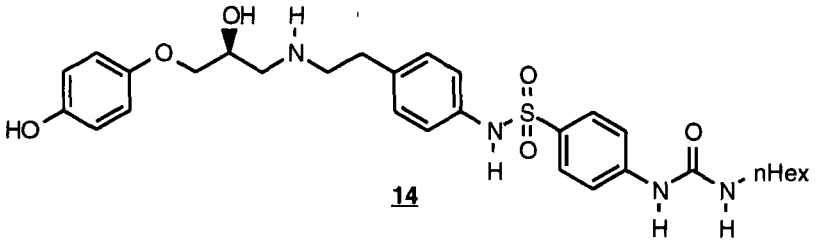


13

Hydroxyphenoxyethanolamine L-755,507 (14), in which a benzenesulfonamide replaces the acid functionality found in many β3 AR agonists, is among the most potent and selective aryloxypropanolamines reported to date, with an EC_{50} of 0.43 nM (IA 0.52) and >440-fold selectivity over binding to β1 and β2 ARs (55). This compound is a weak partial agonist at the β1 AR (EC_{50} = 580 nM, IA 0.25), but shows no efficacy at the β2 AR at 10 μM (55).

Arylethanolamine Derivatives - A number of new derivatives have been identified which contain the 3-chlorophenyl left hand side found in the early β3 AR agonists (4) and (5). In BMS-187413 (15), the negative charge of the sulfonic acid is believed to enhance its selectivity for the β3 AR by decreasing affinity for β1 and β2 ARs (56). This 60 nM



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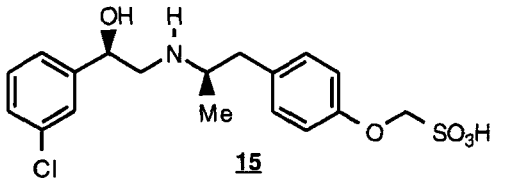
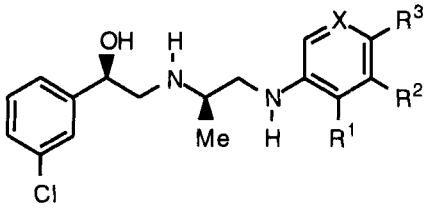


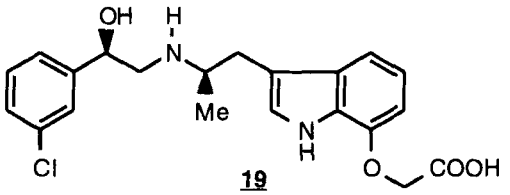
15

partial agonist (IA 0.57) has Ki's at β1 and β2 of 7600 and 2250 nM, respectively. A series of diamine derivatives were found to be more potent β3 AR agonists than isoproterenol (57–59). Carboxylic acid **16** is five-fold more potent (57), while both sulfonic acid **17** (58) and pyridineacetic acid **18** (59) are ten-fold more potent. The latter compound also protects against indomethacin-induced gastric lesions in the rat, with an ED_{50} of 0.001 mg/kg (59).



	R^1	R^2	R^3	R^4
16	F	F	CO_2H	CH
17	H	H	$CH_2CH_2SO_3H$	CH
18	H	H	CH_2CO_2H	N

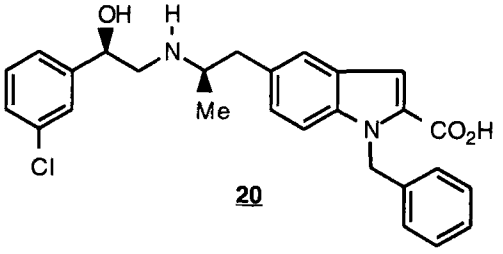
Optimization of an indole lead gave AD-9677 (**19**) (60). This derivative is a potent agonist of both the human (EC_{50} = 0.062 nM) and rat (EC_{50} = 0.016 nM) β3 ARs, with little agonist activity against the cloned β1 and β2 ARs (61). Following chronic (14-day) administration to KK-Ay and db/db mice, a trend toward inhibition of weight gain was observed and significant reductions in plasma glucose, fatty acids, and triglycerides were seen. In the KK-Ay mice, insulin levels were normalized (62).



19

Another indole-derived β3 AR agonist, CP-209,129 (**20**), was discovered in a study directed toward conformationally restricting the phenoxyacetic acid portion of **4** (63). This compound, a partial agonist at the human receptor (IA 0.56), has a β3 EC_{50} of 20

μM, where EC_{50} is defined as the concentration at which the compound achieves 50% of the isoproterenol response. It did not activate the human β1 or β2 ARs at a concentration of 30 μM. A related analog, CP-331,679 (**21**), which contains an aminopyridine left hand side, is a full agonist at the human β3 AR (EC_{50} = 300 nM) with >100-fold selectivity over efficacy at the β1 and β2 ARs (63). Development of this compound was precluded by poor oral bioavailability (<10% in rats) of both it and its corresponding ethyl ester (63).



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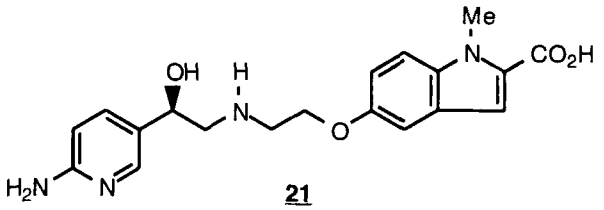
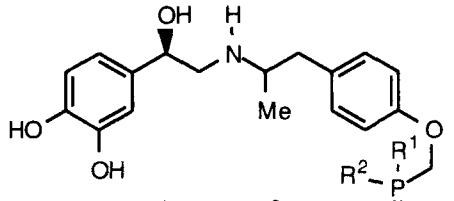
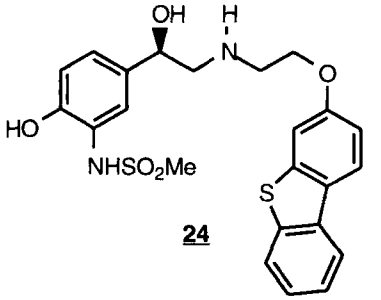


21

Phosphorous-based analogs have been developed in both the aryloxypropanolamine and arylethanolamine series. Phosphonic acid **22** and ester **23** have EC_{50}'s at the human β3 AR of 540 and 460 nM, respectively (64). Dibenzothiophene derivative **24** is reported to be a highly potent, subnanomolar β3 AR agonist (65).

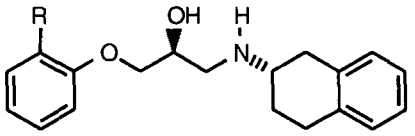


22 (R^1 = OH, R^2 = Ph)
23 (R^1 = OEt, R^2 = Et)



24

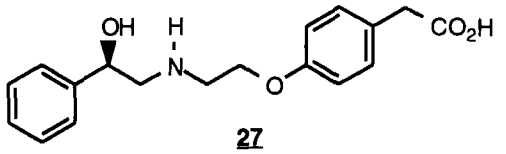
β3 AR Antagonists - SR 58894A (**25**) and SR 59230A (**26**) are the first selective β3 AR antagonists reported, with pA2 values of 8.06 and 8.76, respectively, against rat colon relaxation induced by **6**; and they are 100-1000-fold selective for β3 over β1 and β2 ARs (66). The latter derivative also blocked cAMP production induced by **6** in rat BAT membranes (pK_B = 8.87) (67). Antagonist **26** was also shown to inhibit relaxation of human colonic circular smooth muscle induced by isoproterenol in the presence of



25 (R = $CH_2CH{=}CH_2$)
26 (R = Et)

β1 and β2 AR blockade with a pA2 of 8.31 (68); however, no selectivity data in humans has been reported.

Status of β3 AR Agonists in Development - Of the early β3 AR agonists discovered using rodent models, only **6** remains in clinical development (69,70). This compound is reportedly undergoing Phase II trials for obesity, type 2 diabetes (69) and irritable bowel syndrome (70), and Phase I trials for depression (69). It was well tolerated at doses of 20-180 mg, and an increase in free fatty acids was seen at the highest dose (71). Development of both **2** (72) and ZD-2079 (**27**) (73) was discontinued due to lack of efficacy in Phase II trials. While a 10% increase in resting energy expenditure was seen in humans following a 150 mg dose of CP-114,271 (**28**), desensitization of the response was noted in a multiple dose study (63). Because **28** was subsequently shown to have very weak agonist activity at the human β3 AR, it is likely that these affects are the result of β1 AR stimulation followed by downregulation. This compound is no longer in development (63). β3 AR agonists BMS-194449, BMS-196085, and BMS-201620, which were discovered using a cloned human receptor assay, are reported to be in Phase I (74). The structures of these transdermally active agents have not been published.



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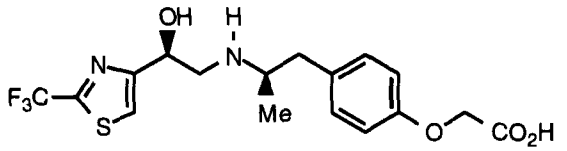

28

Potential New Clinical Targets - A number of new clinical applications for β3 AR agonists have been proposed. Agonist **2** was shown to lower intraocular pressure in glaucomatous monkeys and thus has been patented for the treatment of ocular hypertension (75). This compound has also been shown to reduce body weight loss in rats due to disuse conditioning and may be useful for treating wasting conditions (76). The treatment and prevention of urinary disorders including pollakiuria and incontinence with a β3 AR agonist has been claimed (77). This potential use is supported by evidence that β3 AR mRNA is expressed in human urinary bladder detrusor tissue (78). The expression of β3 AR mRNA in human prostate (10) has led to the proposed use of β3 AR agonists in the treatment of prostate disease (79). Though not a new proposal, the potential use of β3 AR agonists in GI disorders has been recently reviewed (80). The application of β3 AR agonists for the treatment of depression, which was based on the efficacy of **6** in a number of rodent models of this disease (81), appears not to be general. While **6** and its active acid metabolite cause increases in cAMP in rat frontal cortex, several other β3 AR agonists including **4** and **1** are inactive (82). There are conflicting results concerning the presence of the β3 AR in the brain (10,81,83), and **6** may exert its affects through stimulation of the β1 AR (82).

Conclusions - While progress has been made toward understanding the β3 AR's function and role in obesity, it is still not clear whether treatment of humans with a β3 AR agonist will result in sustained weight loss and improvements in co-morbidities. The identification of potent and selective agonists of the human receptor with appropriate pharmacokinetic properties will be necessary before these questions can ultimately be answered in the clinic.

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Chapter 20. New Treatments for Arthritis

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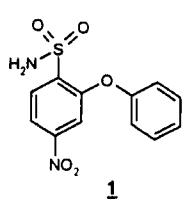
Introduction - Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology associated with bone and cartilage destruction leading to gross, crippling joint deformities and increased mortality. A successful therapy would reduce inflammation and pain and retard or reverse joint destruction and deformity. There are no therapies which effectively accomplish these objectives. Current treatments provide only partial control; non-steroidal anti-inflammatory drugs (NSAIDs) and steroids are still the standard first line treatments. Second line agents have a slower onset of action and are intended to have a disease-modifying effect, hence the term DMARDs. Examples are penicillamine, gold compounds, sulfasalazine, hydroxychloroquine and methotrexate. Often, several drugs are tried, many times in combination and recently, cyclosporin A has been included in trials of combination therapies. However, despite extensive efforts, most patients give up on these treatments and go on to develop crippling disease. A number of new targets and approaches will be discussed in this chapter. Control and therapy of osteoarthritis (OA) presents an even greater challenge than RA, with little in the way of disease-modifying strategies in place to retard the progress of cartilage and bone destruction.

INHIBITORS OF ARACHIDONIC ACID METABOLISM

Cyclo-oxygenase Inhibitors - The therapy limiting side effects of classical NSAIDs, which inhibit both COX-1 and COX-2, are gastric irritation/ulceration and renal damage. Inhibition of the inducible cyclo-oxygenase (COX-2) which is upregulated at inflammatory sites, without affecting the constitutive enzyme (COX-1), which is associated with homeostatic prostanoid synthesis, is a promising approach for the development of safer NSAIDs for the treatment of both RA and OA. Recent COX-2 reviews have appeared covering the therapeutic rationale and chemistry (1,2), patent literature (3) and initial clinical results (4). Selective COX-2 inhibition has been described for several methanesulfonamides (nimesulide, **1**) and analogs of known COX-1 selective inhibitors (3). Several large clinical studies with **1** in OA patients have demonstrated efficacy comparable to NSAIDs and reduced gastrointestinal side-effects (5). Three COX-2 selective triaryl sulfonamides, SC58635 (celecoxib, **2**), MK-966 (**3**) and JTE-522 (**4**), are currently in late phase clinical trials (4,6). Compounds **2** and **3** have been shown to be safe and effective in relieving the signs and symptoms of RA. Importantly, clinical studies showing improved gastrointestinal tolerability have been reported for several currently marketed NSAIDs (nabumetone, meloxicam and etodolac), which appear to be less COX-2 selective than **2**-**4** (7). Further long term studies with the more selective COX-2 inhibitors (**2**-**4**) are needed to define the potential therapeutic utility and advantages of these agents relative to current therapy.



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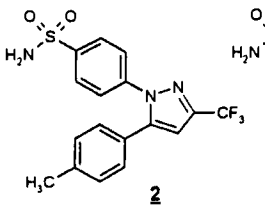

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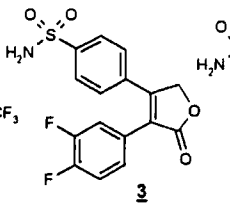

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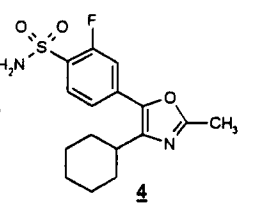

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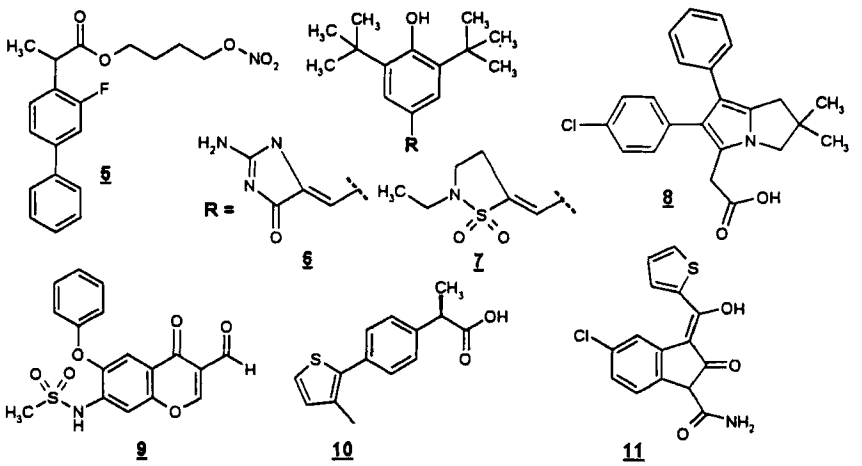
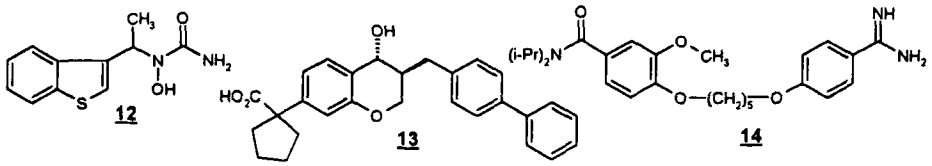
Nitric oxide (NO) exerts beneficial effects on the mucosa similar to prostaglandins (PGs) by modulating mucosal blood flow, mucus release and repair of mucosal injury, and hence NO could counteract the NSAID-induced reduction of cytoprotective PGs. This concept is the basis for the NO-releasing NSAIDs which demonstrate reduced GI damage in repeat dose animal studies relative to the parent NSAID and show evidence for enhanced healing of established ulcers (8). The butoxynitric ester of flurbiprofen (HCT-1026, **5**) was well tolerated in a phase I trial and additional studies are ongoing (9). The phenolic antioxidants (CI-1004, **6** and, S-2474, **7**) and the pyrrole acetic acid (ML-3000, **8**) are dual 5-LO/COX inhibitors (10,11,12). Preclinical evaluation of **6**, **7** and **8** has demonstrated reduced GI damage and enhanced efficacy relative to NSAIDs and clinical studies are ongoing (13,14,15). T-614 (**9**) is a selective COX-2 inhibitor structurally related to **1**. However, both **9** and **8** also inhibit pro-inflammatory cytokine biosynthesis, suggesting the potential for disease-modifying effects in RA (16,17). **9**, which is now being developed as a DMARD, is active in animal models of arthritis and has shown evidence of efficacy in RA (18,19). M-5011 (**10**), although not reported to be a COX-2 selective NSAID, demonstrated reduced ulcerogenic activity in rats and is in phase II trials (20). Tenidap (**11**) a non-selective COX inhibitor lowers plasma levels of acute phase proteins, a profile which clearly distinguishes it from other NSAIDs. At the doses used in RA trials **11** was associated with a proteinuria and a decrease in bone mineral density (21).



Leukotriene Synthesis Inhibitors and Antagonists - Although antagonists and inhibitors of the peptidoleukotrienes (LTC_4, LTD_4), long implicated in pulmonary disease, have demonstrated utility in asthma, their potential in the treatment of arthritis remains unclear (22). A study in RA patients with zileuton (**12**), the first selective 5-LO inhibitor approved for the treatment of asthma, demonstrated some relief of signs and symptoms (23). CP 105,696 (**13**) and CGS-25019C (**14**), antagonists of the potent neutrophil chemotactic agent LTB_4, were effective in the mouse collagen model of arthritis (CIA) (24). RA is the reported target indication for **14**. Initial clinical studies demonstrated complete inhibition of *ex vivo* LTB_4 at the 300 mg dose.



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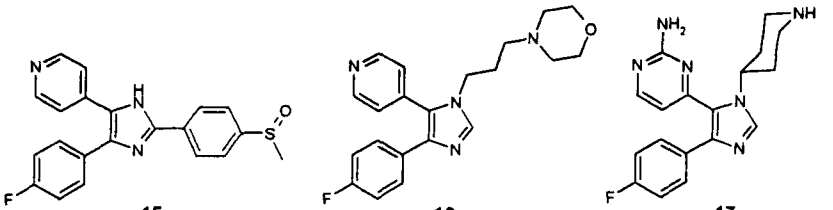
CYTOKINES: INHIBITION AND THERAPEUTIC POTENTIAL

Overproduction of the cytokines TNF-α and IL-1 causes a destructive immune response associated with many inflammatory diseases including RA, OA, multiple sclerosis, lupus, asthma and inflammatory bowel disease and thus these diseases are useful targets for therapy. Other cytokines are clearly involved and play a multitude of roles in inflammation and disease processes. However, there is considerable evidence to show that inhibition of IL-1 and/or TNF-α can have dramatic effects on progression of RA, and intense effort is currently focussed on inhibiting TNF (25).

p38 MAP Kinase Inhibitors - Inhibitors of the serine/threonine p38 MAP kinase (also known as CSBP, SAPK-2, RK) were initially demonstrated to block IL-1 and TNF production in lipopolysaccharide stimulated monocyte/macrophages (26). SB 203580 (**15**) is a highly selective inhibitor of p38 MAP kinase (27), competing with ATP for binding to p38 (28). X-ray crystallographic studies which have elucidated a uniquely shaped binding pocket offer a potential explanation for the selectivity of these inhibitors (29,30). Using **15** as a tool compound, the involvement of p38 in stress response signaling has been probed and many additional consequences have been ascribed to p38 activation/inhibition. Compound **15** has therapeutic activity in collagen-induced arthritis (CIA) as well as the adjuvant arthritic (AA) rat where it has disease-modifying activity indicated by protection of joint integrity (31). SB 210313 (**16**) and SB 220025 (**17**) have also been described as orally active selective p38 inhibitors (32,33).



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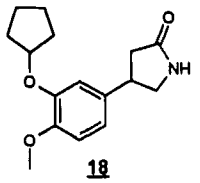
Phosphodiesterase (PDE) Inhibitors - Inhibition of PDE4 results in increases in intracellular levels of adenosine 3'5'-cyclic monophosphate (cAMP). A functional consequence of this increase in LPS-treated monocytes/macrophages is inhibition of TNF-α production (34,35). Both specific (rolipram; **18**) and non-specific (pentoxifylline; **19**) inhibitors of this PDE isoform inhibit TNF and considerable focus has been applied to the synthesis of novel compounds for the treatment of asthma (36) as well as other diseases in which TNF plays a role such as AIDS and RA. Several PDE4 inhibitors such as **18**, **19**, and CP-77059 (**20**) have shown activity in preclinical models of arthritis and **19** is being evaluated in the clinic in RA (37,38). A compound that may be useful in the treatment of RA is CP-353164 (**21**), one of a series of biaryl-carboxamides, that is a potent inhibitor of TNF and PDE4 (39).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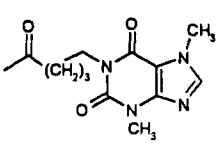
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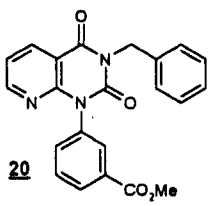
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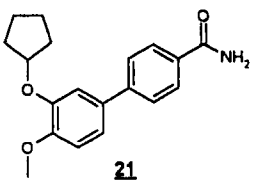
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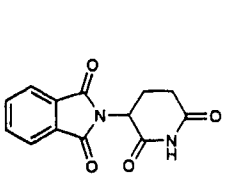
Phthalimides - Thalidomide (22), originally used as a sedative, has immunomodulatory and anti-inflammatory effects. Due to its ability to inhibit TNF-α, the compound is being reevaluated in a number of diseases including Crohn's disease, multiple sclerosis, HIV infection as well as RA. In RA patients, although some positive responses were noted, the side effect profile may be the limiting factor and low doses need to be evaluated to minimize toxicity (40,41). Thalidomide analogs (for example, CC1069, 23) lacking the hydrolytically unstable glutarimide ring are several hundred fold more potent as inhibitors of TNF. They are selective inhibitors of PDE4 and may be better tolerated than other PDE4 inhibitors tested in the clinic.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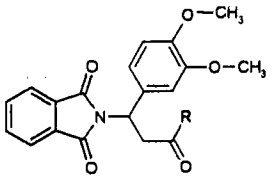
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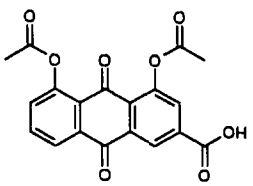
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Diacerhein -IL-1 Inhibitor - Diacerhein (24), the acetylated form of the anthraquinone, rhein, is currently marketed as an antiarthritic in France. In a dog model of OA some beneficial effects have been observed (42). However, in an accelerated version of the same model no statistically significant effects were observed (43).

Biological Agents - Several biological entities designed to inhibit cytokines such as TNF or IL-1 are being evaluated in clinical trials. Enbrel™ comprises a soluble TNFp75 receptor linked to the Fc portion of human IgG1 molecule. Phase III trials have recently been completed and treatment was both effective and safe (44,45). Another fusion molecule is Lenercept (tenefuse - RO 45-2081), a TNFp55-IgG1 molecule that was in Phase II. Efficacy was observed with this molecule, however, antibody responses developed in some patients (46) and its development has been terminated. In addition to the fusion molecules, anti-TNF-α MoAbs are being tested for their potential. Phase III trials have been completed with a chimeric MoAb to TNF-α (Avakine /infliximab - formerly cA2) in RA patients. Therapy, either alone or in combination with methotrexate (MTX), was effective and provides a novel strategy for long-term treatment of RA (47). Efficacy has also been observed with Bay-103356 (CDP-571) which is a humanized anti-TNF (48). An antibody directed to the IL-6 receptor which is active in CIA (49) is under investigation and anti-CD23 is also being developed as an antirheumatic as well as for asthma, allergic rhinitis and other allergic conditions. Soluble CD23 regulates the generation of TNF and IL-1 from human monocytes and efficacy has been demonstrated in CIA, suggesting that neutralization of CD23 may have therapeutic value (50). An antibody that binds with high affinity to B7, a molecule which binds to the CD28 T-cell receptor and co-stimulates T cell proliferation, is in clinical testing for GVHD and RA as an immunosuppressant (51,52).

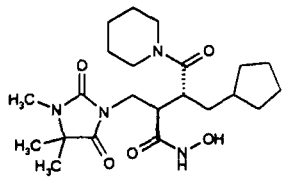
Cytokine Receptor Antagonists - Antril (IL-1ra) is an IL-1 receptor antagonist that is currently in clinical trials for RA. It has been shown to be well tolerated and produce

clinical improvement over a 48 week period (53). The agent is currently in a Phase I/II trial with methotrexate in treating RA. An IL-6 receptor antagonist (SANT 7) is being evaluated for the treatment of RA, as well as multiple myeloma, B lymphomas, Crohn's disease, SLE and psoriasis (54). In preclinical studies in CIA, IL-6 has been shown to be required for disease development (55).

Cytokine Therapy -The Th2 cytokines IL-4, IL-10 and IL-13 inhibit Th1-cell proliferation and oppose the effects of IFN-γ on macrophages (56). This regulation, occurring between Th1 and Th2 cells, is beginning to be exploited for the treatment of RA (57). In preclinical studies the Th1 cytokines IL-4 and IL-10 have been found to have anti-inflammatory and immunosuppressive effects (58) and to be active in arthritis models (59,60). Moving this concept to the clinic, recombinant IL-10 is being tested in patients with RA and a trend for improvement in symptoms has been observed (61).

MATRIX METALLOPROTEINASES (INHIBITORS)

MMPs are zinc-dependent endopeptidases with an important role in degradation and remodelling of extracellular matrix. Many MMP inhibitors have a dual action and also inhibit TNFα. Their role in the degradation of cartilage is of interest as inhibitors of MMPs could well be useful in the treatment of RA and OA (62). However, none is in development for RA at the present time. A series of novel, potent, selective inhibitors of collagenase have been synthesized with good solubility and oral bioavailability (63). Ro32-3555 (**25**) is being developed as a cartilage protective agent for OA and RA (64,65). CGS 27023A (**26**) is an orally active MMP inhibitor active in preclinical models of the disease (66,67). L-758354 (**27**) is an orally active MMP inhibitor with nanomolar activity against stromelysin-1. Although inactive in the AA rat and mouse CIA it has activity in a rabbit model of cartilage destruction induced by stromelysin-1 (68). Tetracyclines and the derivatives doxycycline and minocycline have anti-inflammatory properties unrelated to their antimicrobial activity (69) but which may be related to their ability to inhibit MMPs (70). This activity has led to their evaluation in RA. After taking minocycline for three years, 15 of 23 patients showed a 75% improvement in symptoms , compared with 13% of those who received placebo (71).



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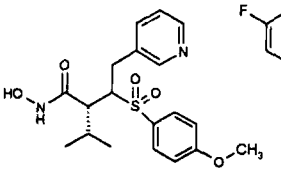

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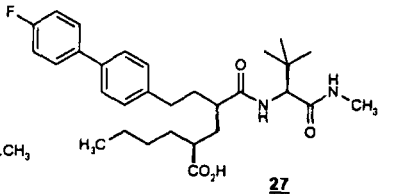

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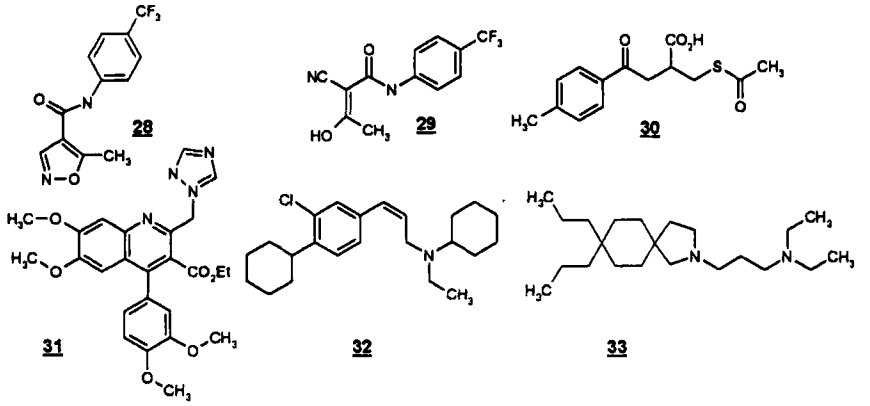
IMMUNOSUPPRESSIVE AND IMMUNOMODULATORY AGENTS

Anti-proliferative compounds - Leflunomide (HWA-486, **28**) is a heterocyclic oral prodrug of the active compound A771726 (**29**). The compound inhibits dihydro-orotate dehydrogenase, thereby disrupting DNA synthesis in immune cells (72, 73). In Phase III trials in RA patients **29** caused an eight-point reduction from baseline in mean tender joint count after three months (74). KE-298 (**30**) has been shown to suppress arthritis in animal models by inhibiting the production of proinflammatory cytokines. It suppresses the TNF-α stimulated production of several MMPs without effect on TIMP-1 (tissue inhibitor-1 of MMPs) (75, 76). Compound **30** was associated with significant improvement in a phase II trial in RA patients and is currently in phase III trials. TAK-603 (**31**) is a new quinoline derivative in clinical development for

treatment of RA and OA. In preclinical studies **31** was effective in rat AA where it suppressed Th1-type cytokine production and protected bone and cartilage from destruction (77,78). SR 31747 (**32**) is an immunosuppressive sigma ligand for RA/autoimmune disease. **32** Inhibits T cell proliferation *in vitro*, graft-versus-host disease and DTH in mice (79) and blocks *ex vivo* LPS induced production of IL-1, IL-6 and TNF in a dose-dependent manner (80).

Macrophage Targeting Agents - Atiprimod (SK&F 106615) (**33**) is an azaspirane being developed as an immunomodulatory macrophage-targeting agent (81). It has therapeutic activity in animal models of autoimmune disease and transplantation (82) and is particularly effective in the AA rat model where it inhibits inflammation and protects joint integrity (83). Phase I clinical studies in RA patients are complete with no adverse side effects observed (84). Unlike many DMARDs, atiprimod is minimally immunosuppressive (85) and host defenses against infection are not compromized by the azaspiranes (86). Mechanistically, the azaspiranes accumulate in macrophage lysosomes and alter the inflammatory activity of these cells (82, 87).



Macrophage Inhibitory Factor (MIF) was first described as a soluble factor released from antigen sensitized lympocytes. It has now been described as a phenylpyruvate tautomerase (88) and acts to override the anti-inflammatory activity of steroids. Anti-MIF antibody has anti-inflammatory activity in the AA rat (89) and may have utility in the treatment of a broad range of inflammatory and autoimmune disease.

Induction of Tolerance - In the search for therapies that are more specific and less toxic than standard immunosuppressive agents, several groups are attempting to induce antigen-specific tolerance by oral administration of antigen. This strategy has proven effective in a number of animal models of autoimmune disease and is now being tested in the clinic (90, 91, 92). A study with bovine collagen proved somewhat disappointing in that statistical differences were not found (93). However in a recently reported multicenter study, utilizing the cumulative Paulus criteria for response, chicken type II collagen (Colloral) at 20 ug/day was shown to be superior to placebo.

Vaccines - The observation that T-cell receptor gene use is observed in animal models of autoimmune disease has led to T-cell receptor peptide therapy in RA. An example is IR 501 which consists of a combination of three T cell receptor peptides plus adjuvant as a vaccine. Significant improvement compared to placebo was observed with low doses (90ug) (94). AnervaX™ is a peptide vaccine that targets the DR4 and DR1 HLA (human leukocyte antigen) molecules found in 90% of RA patients. The vaccine has shown efficacy In Phase II clinical trials and no generalized immunosuppression was observed (95).

Human Cartilage Glycoprotein-39 - HC gp-39 is an antigen that is recognized by T cells from RA patients. T cell immunity against HC gp-39 in mice is associated with arthritis and the gp-39 protein has the potential to block arthritis in a mouse model of arthritis (96), making this antigen a candidate for antigen-specific immunotherapy.

Targeting T-cell Subsets - Following the identification of CD4 and CD8 T cell subsets (97), targeting of these subsets to inhibit their function, or deplete them, became a therapeutic strategy in transplantation. The concept is now being applied to the treatment of RA. MoAbs against the CD4 subset are being evaluated by a number of groups with the aim of inhibiting function without cell depletion. Two MoAbs, SB 210396 (PRIMATIZED® IDEC-CE9.1 - Keliximab) and SB 217969 (PRIMATIZED® IDEC-151 - Clenoliximab) are being evaluated in the clinic. Clenoliximab has been selected as the lead antibody of the two for the treatment of RA (98). Both antibodies seek to regulate the function of CD4 cells and SB 210396 treatment resulted in significant responses in RA patients (99, 100). 4162W94 is a humanized non-depleting anti-CD4 MoAb that has shown improvement in disease parameters (101). Another non-depleting anti-CD4 MoAb (Orthoclone, OKTcdr4a) affects T cell functions with selective decreases observed in IL-2 and IFN-γ production, indicating an effect on the Th-1 subset (102). MEDI-507, is a humanized MoAb which binds specifically to the CD2 antigen receptor on T cells and natural killer cells; preclinical studies indicate a long lasting inhibitory effect of this antibody on T cell responses *in vitro* (103).

Gene therapy - By delivering genes encoding a therapeutic agent to the joint, elevated levels of the agent can be produced without the systemic side effects often associated with current treatments. Thus, elevated levels of a beneficial cytokine or an antagonist of a pro-inflammatory cytokine could block events leading to erosion and inflammation. This approach is being evaluated in animal models. In CIA, gene therapy with IL-10 has been shown to be effective (104, 105). Overexpression of IL-12 exacerbated disease while gene transfer of IL-4 or TGF-β diminished disease (106). Preclinical studies with transfer of a cDNA encoding the IL-1ra has been shown to suppress disease activity in animal models and is now being used in humans (107, 108).

FUTURE STRATEGIES

The search for new therapeutic agents for RA continues to be a difficult and arduous endeavor which is compounded by the unknown etiology of the disease and thus the inability to target the causative agent. The discovery of the role of cytokines in the disease is opening up the area of specific gene therapy. New genomic technology such as that provided by differential display of genes that are up or down-regulated will facilitate the discovery of new targets for drug design. Areas not exploited to their full potential such as regulation of apoptosis and angiogenesis offer the potential of new therapies. As the role of Th1 and Th2 cytokines is resolved, adjusting the balance in RA could have beneficial effects. Identifying those patients predisposed to RA or OA will allow attack on the disease at a much earlier stage than has been previously been possible. Other targets such as complement, bradykinin, chemokines, TNF-α ribozymes and antisense molecules are yet to be fully exploited.

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Chapter 21. Recent Advances in the Development of Agents for the Treatment of Type 2 Diabetes

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Introduction - The Centers for Disease Control and Prevention recently reported that the incidence of diabetes in the US is at an all time high with 15.7 million Americans thought to be affected. There are 120 million diabetics worldwide and this number is estimated to increase to greater than 200 million by 2010 (1). Type 2 diabetes accounts for more than 90% of these cases. Many cases of type 2 diabetes are undiagnosed primarily because the early stages of the disease are asymptomatic. Type 2 diabetes is defined by high plasma glucose levels and is characterized by peripheral insulin resistance, insufficient insulin secretion by the β-cells of the pancreas and increased hepatic glucose production (2). Insulin resistance seems to occur prior to the development of overt diabetes and requires a diminution in the function of insulin secreting β-cells before the hyperglycemia typical of the disease becomes manifest (3). Type 2 diabetes is a result of genetic predispositions coupled with environmental factors such as obesity and a lack of exercise (4). The cost of diabetes is reflected in its complications of retinopathy, nephropathy, neuropathy and macro- and microangiopathy (5). These complications can result in blindness, kidney failure, limb amputation and cardiovascular disease. Most diabetics die of cardiovascular disease.

The Diabetes Control and Complications Trial showed that better glycemic control could delay the onset and slow the progression of diabetic complications in type 1 patients (6). The UK Prospective Diabetes Trial is studying the relationship of glycemic control and complications in type 2 diabetics and is due to report in 1998 (7). In 1997, the WHO and the American Diabetes Association recommended that the level of fasting plasma glucose required for diagnosis of type 2 diabetes be lowered from 140 to 126 mg/dL (8). An additional recommendation was that the term Non-Insulin-Dependent Diabetes Mellitus (NIDDM) no longer be used as a classification of the disease. The FDA has recently issued draft guidelines entitled "Evaluation of New Treatments for Diabetes Mellitus" (9). These guidelines cover clinical trials for new antidiabetic drugs and suggest that a total of 2,000 patients need to be exposed to the drug with an adequate number of patients exposed for one year in controlled trials. The FDA still accepts Hemoglobin A_{1c} (HbA_{1c}) as a measure of chronic hyperglycemia and considers a decrease of 0.7% units for the last 6 months of a yearlong study to be clinically significant. HbA_{1c} is a glycated form of hemoglobin and is a marker of average plasma glucose concentrations over about 2 months (10).

From a mechanistic viewpoint, most therapies for the treatment of Type 2 diabetes can be grouped into one of four categories: insulin or insulin secretagogues; enhancers of insulin action; inhibitors of hepatic glucose production or inhibitors of glucose absorption. This chapter will focus on therapies for type 2 diabetes which have glucose-lowering activity.

Insulin - The use of insulin in type 2 diabetics was recently evaluated in a large clinical study (11). After one year of treatment with insulin, HbA_{1c} levels were decreased by 0.9% versus controls. A new rapid-acting insulin analog, [Lys(B28),Pro(B29)]-human insulin (LYSPRO) has been developed (12). A clinical study in type 1 and type 2 diabetics reported lower postprandial glucose levels for those patients using LYSPRO vs. those using regular human insulin (13). A number of alternatives to injecting

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insulin are being investigated including oral insulin (14), inhaled insulin (15) and gene therapy (16).

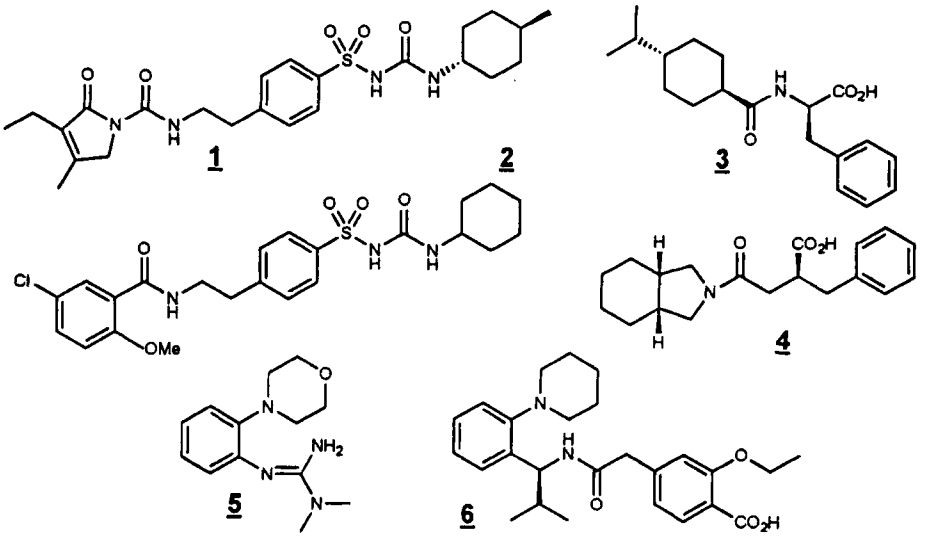
INSULIN SECRETAGOGUES

The Sulfonylurea Receptor - Insulin secretagogues of the sulfonylurea class have been the mainstay of oral therapy for type 2 diabetes for many years. It has been known for some time that these agents stimulate insulin secretion by blocking an ATP-sensitive K^+ channel (KATP) in the pancreatic β-cell leading to membrane depolarization, elevation in intracellular Ca^{2+} and insulin granule exocytosis. Recently, new insight into the mechanism of action of this class of compounds has been gained following the cloning and expression of its molecular target, the sulfonylurea receptor (SUR). A thirteen membrane spanning protein of the ATP-binding cassette superfamily, the SUR multimerizes with an inward rectifier type K channel (kir 6.2) to form an ATP-sensitive K channel. This area has been recently reviewed (17,18).

New Insulin Secretagogues - Two types of new secretagogues have recently appeared on the market or are in late-stage development: a new-generation sulfonylurea and short acting non-sulfonylureas that interact with KATP. Glimepiride (Amaryl, **1**) is the new generation sulfonylurea having a short onset of action and a long duration of action (19). Although photoaffinity studies with **1** have suggested a different binding site on KATP from that of the older sulfonylurea glibenclamide **2**, their effects on KATP are very similar (20,21). Clinical studies have also failed to demonstrate any obvious differences between the glucose-lowering profiles of **1** and **2** under euglycemic or hyperglycemic conditions (22,23). Four non-sulfonylurea insulin secretagogues which block KATP are in advanced clinical development: A-4166 **3**, KAD-1229 **4**, BTS 67 582 **5** and repaglinide (Prandin, **6**). These new agents have a rapid onset and short duration of action, and may improve glycemic control without inducing prolonged hyperinsulinemia that has been associated with weight gain and hypoglycemic episodes. In animal studies, the hypoglycemic effect of **3** was rapid and short-lasting, a profile consistent with rapid absorption and clearance of the compound (24-26). **4** caused a dose-dependent reduction in glucose in fasted beagles with a maximal effect at 1h (27). **4** may have additional metabolic effects in the liver (28,29). **5** is not as short acting as the above two compounds, with glucose lowering effects still detectable 4h following oral administration. **5** may also have extrapancreatic effects as it causes glucose lowering in the streptozotocin rat where **2** is ineffective. **5** did not displace [3H]-**2** from HIT cell membranes, although its effects were reversed by the KATP channel opener diazoxide, suggesting an interaction of the compound with the KATP channel at a site distinct from sulfonylurea binding (30). **6** was recently approved by the FDA for treatment of type 2 diabetes (32). In a 24 week study, **6** caused improvements in HbA_{1c} in line with those typically seen with sulfonylurea therapy. One-year comparator trials with other insulin secretagogues did not demonstrate a significant reduction in hypoglycemic episodes with **6** (33).

GLP-1 - Glucagon-like Peptide-1 (GLP-1) is reported to be a glucose sensitive insulin secretagogue, to enhance peripheral insulin action, to inhibit gastric emptying, to inhibit glucagon secretion and to be a satiety factor. GLP-1 physiology has recently been reviewed (34). The GLP-1 receptor knockout mouse has mild fasting hyperglycemia and shows glucose intolerance suggesting that GLP-1 plays a role in maintaining normal glucose homeostasis (35). GLP-1 has a very short half life in plasma (< 2 min) which may be chiefly attributed to rapid cleavage by dipeptidyl peptidase 4 (36,37). A number of short-term clinical studies have shown positive results in type 2 diabetics with GLP-1. An overnight infusion of GLP-1 (7-36) amide, the major circulating form of GLP-1 in man, significantly lowered glucose concentrations and normalized insulin response to glucose (38). All clinical studies to date have utilized either subcutaneous

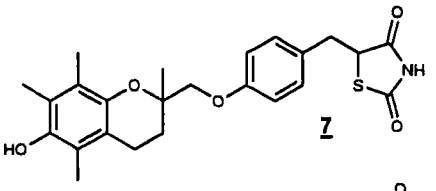
injection of GLP-1 or continuous infusion. Analogs of GLP-1 with enhanced metabolic stability have been reported (39).



ENHANCERS OF INSULIN ACTION

Compounds which decrease insulin resistance have been called insulin sensitizers or enhancers of insulin action. An agent which enhances insulin action should reduce hyperglycemia without a concomitant increase in plasma insulin levels.

Troglitazone and other Thiazolidinediones (TZDs) - The TZDs have been extensively reviewed (40-42). As a class, the thiazolidinedione antidiabetics have been shown to enhance insulin action in a number of animal models of insulin resistance and type 2 diabetes and in man. In clinical studies in type 2 diabetics, TZDs have been shown to improve hyperglycemia while decreasing levels of insulin, triglycerides and free fatty acids (43,44). Troglitazone (Rezulin, 7) is the first and, thus far, the only thiazolidinedione to be approved for the treatment of type 2 diabetes (45). At the end of a a six month study in type 2 diabetics, a 600 mg once daily dose of troglitazone was found to lower fasting serum glucose by 60 mg/dL, HbA_{1c} by 1.1%, insulin by 2.4 μU/mL and triglycerides by 72 mg/dL versus placebo (46). The clinical development of thiazolidinediones has been plagued by side-effect problems such as the liver and cardiac hypertrophy seen in toxicological studies in animals, tumors in animals and small changes in hemacrit in man (42). In a 96 week study in type 2 diabetics, troglitazone was found not to carry a risk of cardiac enlargement or impairment of cardiac function (47). Recently, however, troglitazone, has been associated with rare reports of liver dysfunction (indeed, there was an 2.2% incidence of increased liver enzymes in controlled trials with the drug) (42). Troglitazone has been voluntarily withdrawn from the market in the UK due to concerns about possible adverse effects on the liver but remains on the market in the US and Japan with increased monitoring of liver function in patients (48,49). Two other thiazolidinediones, rosiglitazone (BRL 49653, 8) (50) and pioglitazone 9 (51), are in clinical trials (48).



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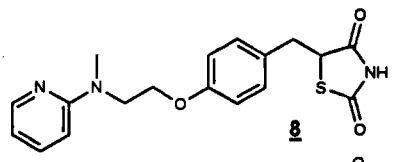

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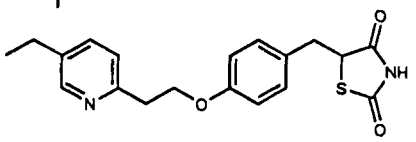

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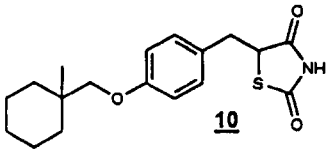

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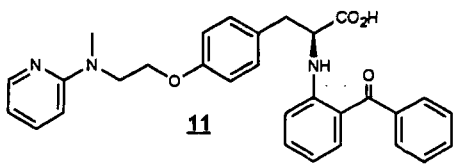
Peroxisome-Proliferator Activated Receptor γ (PPARγ) - The antihyperglycemic effect of the first member of the TZD class, ciglitazone **10** (52), was discovered serendipitously and subsequent TZDs were developed using *in vivo* animal models as the mechanism of action was unknown (40-42). A number of TZDs, however, were reported to induce preadipocytes to differentiate into adipocytes *in vitro* (53,54). In the same time frame, the ligand-activated transcription factor PPARγ was shown to play a major role in adipocyte differentiation (55,56). Subsequently **8** was shown to be a high affinity ligand for PPARγ (57). This was followed by other reports of TZDs having affinity for PPARγ (58-60). Indeed, the affinity of a number of TZDs for PPARγ has been correlated with *in vivo* antidiabetic efficacy in the *ob/ob* mouse (61). While the endogeneous ligand for PPARγ is still uncertain, a number of molecules found in man and other mammals are known to bind to and activate it, including 15-deoxy-$\Delta^{12,14}$-PGJ_2 (60,62) and most dietary fatty acids (63). Several non-TZDs which bind to PPARγ have been shown to have antidiabetic activity like the TZDs in animal models. These include the phenylpropionic acids **11** (64) and SB-219994 **12** (65). The structurally related **13** has antidiabetic activity but its PPARγ activity has not been reported (66).

There are good reviews on PPARγ (67-69). PPARγ is most abundantly found in adipose tissue and the large intestine with lower levels in the liver, kidney, small intestine and skeletal muscle (70). It is known to regulate a number of genes whose products are known to play a role in fat and glucose metabolism such as lipoprotein lipase, malic enzyme and PEPCK (68). It exists as two splice variants, PPARγ1 and PPARγ 2, which share a common ligand binding domain (71).



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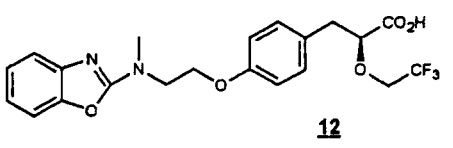

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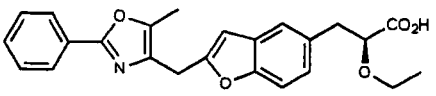

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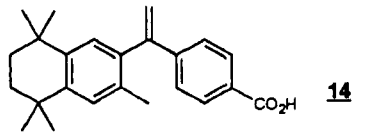

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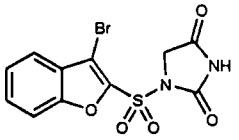
Rexinoids - PPARγ is transcriptionally active as a heterodimer with the retinoic acid X (RXR) receptor. Ligands for this receptor are known as rexinoids. Rexinoids, including LG 1069 **14** have been shown to have antidiabetic activity in *ob/ob* and *db/db*

mice with a profile reminiscent of the TZDs (72,73). In addition, rexinoids and PPARγ agonists were reported to have synergistic activity in *in vitro* cotransfection assays (73). Curiously, this activity has been seen with both RXR agonists and antagonists (74). Thus, it seems that antidiabetic effects can be seen with ligands for both halves of the PPARγ /RXR heterodimer. **14** is being evaluated in the clinic (75).

β3 Adrenergic Receptor Agonists - The potential of this class of compounds are discussed in a separate chapter of this volume.

Miscellaneous Enhancers of Insulin Action - The aldose reductase inhibitor M16209 **15** has been reported to improve insulin resistance and lower plasma glucose in the *ob/ob* mouse (76). BM 13.0913 **16** behaves similarly in this model (77). SDZ PGU 693 **17** is reported to improve insulin resistance in the cynomolgus monkey and is active in the *ob/ob* mouse but is mechanistically distinct from the TZDs (78). No molecular targets for these molecules have been identified.



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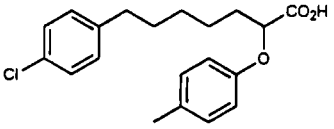

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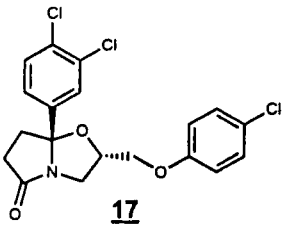

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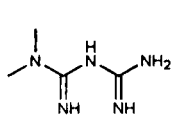
INHIBITORS OF HEPATIC GLUCOSE PRODUCTION

Metformin - Although the biguanide, metformin **18**, has been available for decades in Europe it was only approved in the US in 1995 (79). While the molecular target for this drug has not been identified, its primary mode of action seems to be the inhibition of hepatic gluconeogenesis (80). It is sometimes called an enhancer of insulin action but this may be an indirect effect; it has a distinctly different profile from troglitazone in *in vitro* assays (81). In a clinical study in type 2 diabetics over 29 weeks, metformin given at a daily dose of up to 2.55g was found to lower plasma glucose by 55 mg/dL and HbA_{1c} by 1.5% (82). Metformin has been extensively reviewed (83-85).



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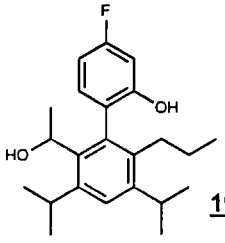

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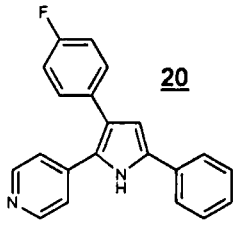

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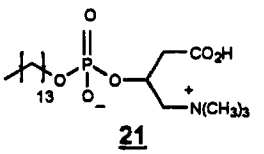
Glucagon Antagonists - The 29 amino acid peptide, glucagon, is secreted by the α-cells of the pancreas and opposes many of insulin's actions. It has been suggested that the enhanced hepatic glucose production seen in type 2 diabetics is due to excess circulating glucagon (86). It had been difficult to validate this hypothesis as 'clean' glucagon antagonists lacking residual partial glucagon agonism were not known. Recently, [desHis^{1},desPhe^{6},Glu^{9}]-glucagon amide was reported to be a 'pure' glucagon antagonist (IC_{50}=48nM) and that it lowered plasma glucose by 60% in a streptozotocin-induced diabetic rat (87,88). Compounds **19** and **20** are claimed as small molecule glucagon antagonists but no functional data has been reported (89,90).

Other Inhibitors of Hepatic Glucose Production - Carnitine palmitoyltransferase I (CPT-1) is the rate limiting step in long-chain fatty acid oxidation. Inhibition of CPT-1 should lead to decreased glucose production by the liver. The phosphate diester **21** is a CPT-1 inhibitor (IC_{50}=3.4μM) which was found to lower blood glucose to near normal levels when given orally to a streptozotocin-induced diabetic rat (91). The chlorogenic acid analog **22** is an inhibitor of glucose-6-phosphatase translocase (IC_{50}=2.5μM) and inhibits glucose output in a perfused rat liver (92).



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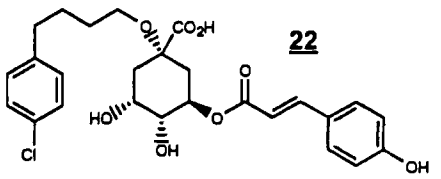

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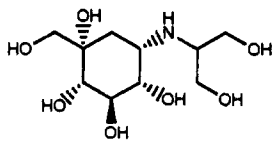
INHIBITION OF GLUCOSE UPTAKE

Molecules in this class slow the absorption of dietary glucose which has the result of diminishing postprandial hyperglycemia. Two mechanisms for achieving this are the inhibition of gastric emptying and the inhibition of intestinal glucosidases.

Glucosidase Inhibitors - Inhibition of intestinal α-glucosidases inhibits the breakdown of oligo- and disaccharides from dietary complex carbohydrates and thus slows the absorption of the glucose they contain (93). Acarbose was the first member of this class to be approved for the treatment of type 2 diabetes (94). Subsequently, voglibose **23** (95) and miglitol **24** (96) have been also approved. Clinical data with acarbose has been reviewed (97). In a 22 week trial, acarbose was shown to decrease HbA_{1c} by 0.78% versus placebo.



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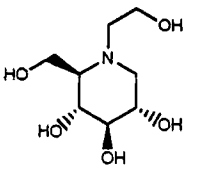

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Inhibition of Gastric Emptying - A number of hormones are known to inhibit gastric emptying including GLP-1, CCK-8 and amylin (98). Pramlintide is an amylin analog with improved physical chemical characteristics which is being investigated in the clinic for the treatment of diabetes (99;100).

MISCELLANEOUS ANTIDIABETICS

A fast-release formulation of the ergot alkaloid and D_2 agonist bromocriptine (Ergoset) was reported to produce a 0.6% decrease in HbA_{1c} in type 2 diabetics after a 24 week trial (101). Bromocriptine's effects on glucose and body weight in *ob/ob* mice can be enhanced with the co-administration of the D1 agonist SKF 38393 (102). Chromium picolinate supplements were shown to have a beneficial effect on plasma glucose and HbA_{1c} in type 2 diabetics (103). Chromium is an essential trace element which plays a role in carbohydrate and lipid metabolism.

FUTURE DIRECTIONS FOR TYPE 2 DIABETES THERAPIES

The six year results of the UK Prospective Diabetes Study suggest that none of the current therapies for type 2 diabetes studied (diet, insulin, glibenclamide or metformin)

are particularly efficacious as monotherapy for long-term control of glycemia (104). A current trend in treating type 2 diabetes seems to be the use of multiple therapies with different mechanisms of action. Recent trials have shown advantage in using metformin plus a sulfonylurea (105) or adding troglitazone to glibenclamide (106).

There are still unmet needs in treating this disease and there are a number of areas where new drugs might intervene particularly in the areas of insulin action and in insulin secretion and preservation of β-cell function.

Insulin Action - Type 2 diabetics have decreased insulin-mediated glucose transport and this may be due to altered regulation of the insulin responsive glucose transporter Glut4 (107). Overexpression of human Glut4 in *db/db* mice led to an improvement in glycemic control (108). The activity and/or level of a number of proteins have been linked to insulin resistance including IRS-1 and 2 (109), PKC isoforms (110) and Akt kinase (111). The cytokine, Tumor Necrosis Factor α (TNFα), has been linked to insulin resistance (112). Neutralization of TNFα in the insulin resistant *fa/fa* rat was shown to improve insulin sensitivity (112) but a similar experiment in type 2 diabetics showed no effect on insulin sensitivity (113). Insulin resistance was decoupled from obesity in transgenic mice lacking the adipocyte fatty acid protein (114). Finally, nitric oxide has been suggested to modulate insulin action in skeletal muscle (115).

β-cell Function – Recent discoveries from human disease linkage suggest new approaches to tackling the progressive deterioration in β-cell function associated with type 2 diabetes. The first gene positively linked with susceptibility to type 2 diabetes encoded the enzyme glucokinase, and was discovered in a sub-population of type 2 diabetic patients, maturity onset diabetes of the young (MODY) patients (116). Two new genes, HNF-1 and HNF-4, hepatic transcription factors that are also expressed in the pancreas, were recently linked with two other forms of MODY (117,118). HNF-4 is thought to contain a ligand binding domain but no ligand has been identified to date (119). A decrease in β-cell mass seems to play an important role in the etiology of diabetes in these sub-sets, suggesting that therapies aimed to increase β-cell proliferation or prevent β-cell apoptosis might be useful approaches to ameliorate or reverse the deterioration in β-cell function. Studies in the Zucker diabetic rat, an animal model of type 2 diabetes, have shown that there is a progressive accumulation of triglycerides in the islets of Langerhans. This accumulation has been suggested to result in a suppression of glucose-induced insulin secretion as well as inducing β-cell apoptosis via an induction of nitrous oxide synthase, the so-called lipotoxicity hypothesis (120, 121). Therapic interventions which decrease islet triglyceride may preserve islet function.

Prevention of Type 2 Diabetes - Impaired Glucose Tolerance (IGT) is a state which can precede overt type 2 diabetes. IGT is characterized by insulin resistance and a reduced capacity to respond to a glucose challenge. The Diabetes Prevention Program is a large trial begun in 1996 looking at the use of lifestyle changes (diet and exercise) or drug intervention (metformin or troglitazone) in IGT people (122). The goal of this trial is to determine whether diabetes can be delayed or prevented in the IGT population.

Summary - Recent advances in type 2 diabetes therapeutic research include the cloning of the sulfonylurea receptor, the identification of PPARγ as the molecular target of the TZDs and the discovery that RXR ligands have antidiabetic activity. The approval of troglitazone is an important advance in the treatment of type 2 diabetes.

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Chapter 22. Prostanoid Receptors

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Introduction - Prostaglandin (PG) E_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TxA_2), collectively known as prostanoids, are derived from arachidonic acid in a two-step enzymatic reaction (Figure 1). The first step of the reaction is the cyclooxygenase activity in which two molecules of oxygen are inserted into arachidonic acid resulting in the formation of the 15-hydroperoxy compound PGG_2. This is followed by the peroxidase activity which results in the reduction of the hydroperoxy group to hydroxy, giving PGH_2. Two different cyclooxygenases (COX) can catalyze the reaction, a constitutive form, COX-1, and a highly inducible form, COX-2 (1). These two enzymes are the targets of non-steroidal antiinflammatory drugs (NSAIDs) and in the case of COX-2, novel selective inhibitors, which block the biosynthesis of the five primary prostanoids enumerated above.

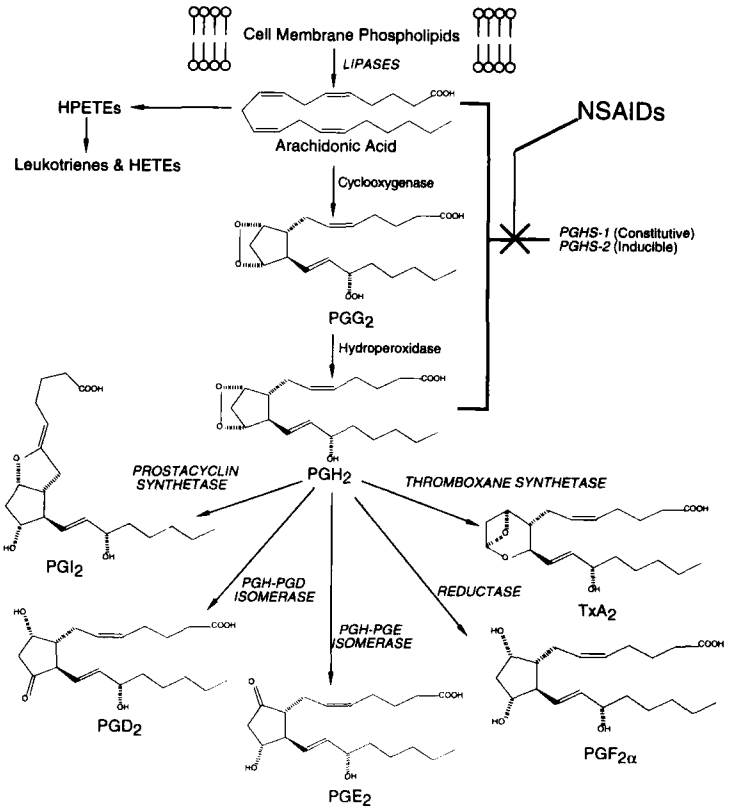

Figure 1. Biosynthetic pathway for the formation of naturally occurring prostanoids.

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These molecules have long been known to behave as important mediators in terms of both physiology and pathophysiology (2). Over the years they have been implicated in a number of areas of therapeutic interest including inflammation and pain, pyrexia, renal disease, cancer, glaucoma, male sexual dysfunction, osteoporosis, cardiovascular disease, preterm labor, allergic rhinitis and asthma (3). However, in most cases the precise role that specific prostanoids play in these different disease processes has not been clearly delineated. Prostanoids which have been or are currently marketed as drugs are shown in Table 1. All of them are either synthetic derivates or analogues of PGE_2, PGE_1 or PGI_2. In addition most are potent but non-selective agonists of the EP subtypes, FP or IP prostanoid receptors with the majority developed prior to the cloning of the prostanoid receptor family (see below).

Table 1. Marketed Prostanoids.

COMPANY	DRUG NAME	THERAPEUTIC INDICATION
Pharmacia & Upjohn	Carboprost trometamol	Abortifacient
Ono	Gemeprost	Abortifacient
Schering AG	Sulprostone	Abortifacient
Searle	Arthrotec	Arthritis
Ono	Limaprost	Buerger's disease
PharmaSciences	Dinoprostone (PGE_2)	Childbirth
Ueno	Isopropyl unoprostone	Glaucoma
Pharmacia & Upjohn	Latanoprost	Glaucoma
Ono	Alprostadil (PGE_1)	Male sexual dysfunction
Vivus	Alprostadil	Male sexual dysfunction
Ono	Alprostadil alfadex	Male sexual dysfunction
Ono	Alprostadil	Peripheral vascular disease
Ono	Alprostadil alfadex	Peripheral vascular disease
Toray	Beraprost	Peripheral vascular disease
Schering AG	Iloprost	Peripheral vascular disease
Glaxo Wellcome	Epoprostenol (PGI_2)	Pulmonary hypertension
Roche	Enprostil	Ulcers
Searle	Misoprostol	Ulcers
Ono	Ornoprostil	Ulcers

The primary prostanoids are potent paracrine and autocrine agents which elicit their effects through interaction with prostanoid receptors[1], members of the G-protein coupled receptor (GPCR) superfamily. Eight prostanoid receptors have been recently cloned and characterized, three of which couple to mobilization of intracellular calcium EP_1 (4), FP (5) and TP (6), four of which couple to an increase in intracellular cAMP levels, EP_2 (7), EP_4 (8), DP (9) and IP (10) and, finally, the EP_3 receptor (11) which couples to a decrease in intracellular cAMP accumulation (see Figure 2). In addition, isoforms have been identified in human for EP_3 (eight isoforms) (12) and TP (two isoforms) (13) which are generated by alternative splicing and vary in the length and amino acid composition of their carboxyl-terminal tails.

[1] Prostanoid receptors are designated following the recommendation of the IUPHAR Commission on Receptor Nomenclature and Classification (56).

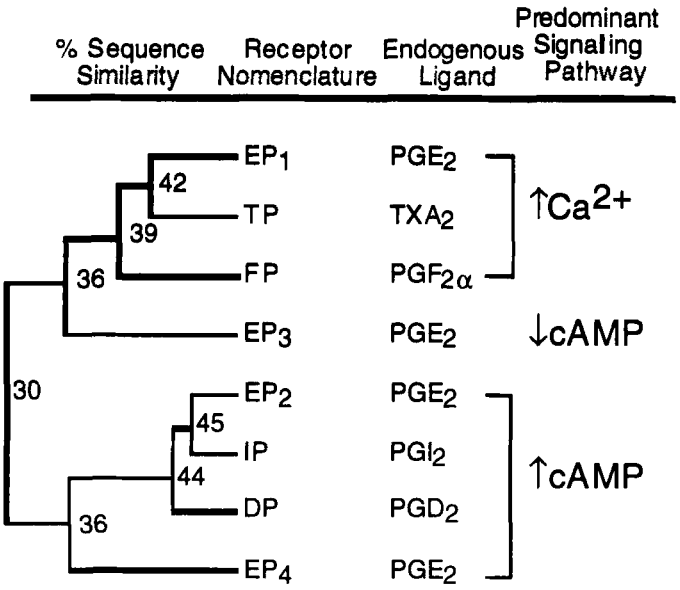
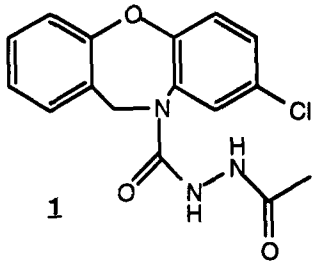


Figure 2. Human prostanoid receptor family.

The molecular characterization of the prostanoid receptors has spawned renewed interest in the prostanoid field. Research is focused on correlating a specific receptor or receptors with different disease pathologies. In addition, the availability of the cloned receptors has already generated interesting data from knockout mice and is a key step in the development of potent and selective prostanoid receptor agonists and/or antagonists for testing in the therapeutic areas outlined above. This chapter will discuss previous and current development of prostanoid receptor agonists and antagonists for various therapeutic indications.

Anti-Inflammatory and Analgesic Agents - The first PG antagonist, SC-19220 **1** was developed using a guinea pig ileum (GPI) smooth muscle contraction assay (14). At the time, the utility of a PG antagonist as a therapeutic agent was unknown. The compound was a weak antagonist with a pA_2 = 5.5. Soon thereafter, the mechanism of action of NSAIDs was elucidated (15) that showed inhibition of COX prevented the formation of prostanoids in general and in particular PGE_2, the putative mediator(s) of inflammation and pain. Subsequently, SC-19220 and SC-25469 **2**, N-substituted dibenzoxapines, were shown to be analgesic agents in two rat models of pain (16). The proposed mechanism of action involved antagonism of PGE_2 at the prostanoid EP_1 receptor according to the classification of prostanoid receptors in smooth muscle (17). More potent compounds from the same structural class, SC-51089 **3** and SC-51234A **4**, with pA_2 = 6.5 and 7.5, respectively, in the GPI assay, showed efficacy in the phenylbenzoquinone writhing assay in mouse (18). SC-51089 was dropped from development due to issues of metabolism (18). The most potent compound in this class was SC-51322 **5** with a pA_2 = 8.1 in the GPI assay and an ED_{50} = 0.9 mg/kg in the mouse writhing assay (19). Taken together these studies suggest that at least the EP_1 receptor plays a role in inflammatory pain.



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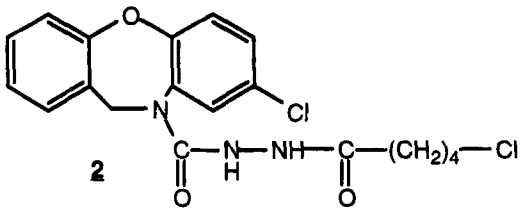

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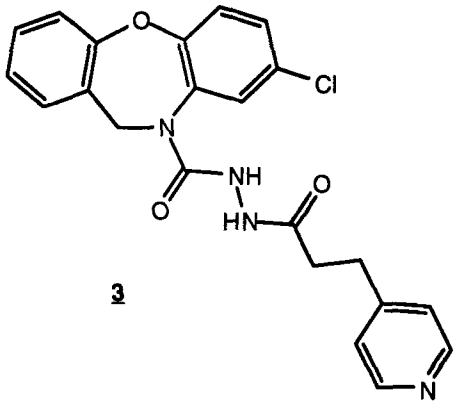

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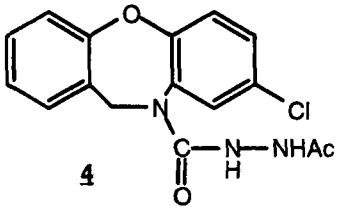

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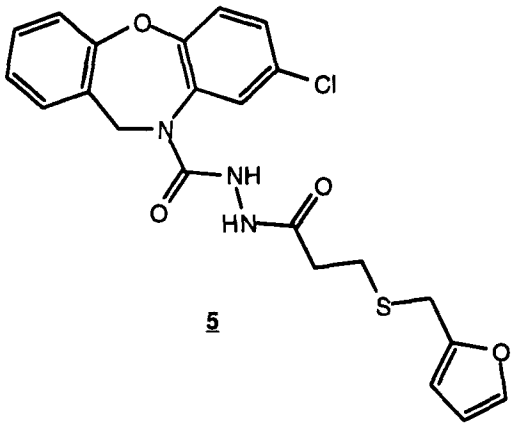

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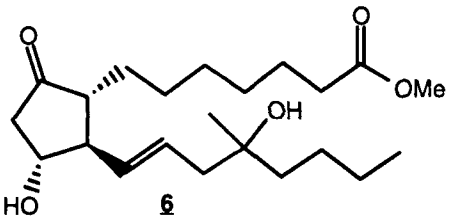
Recently, additional evidence for the involvement of PGE_2 and hence EP receptor subtypes in inflammation and pain has been reported. Specific monoclonal antibodies to PGE_2 (termed 2B5), that neutralize the activity of PGE_2, were efficacious in a phenylbenzoquinone-induced model of nociception (20). Furthermore, these antibodies could reverse established hyperalgesia in a carrageenan-induced hyperalgesia model (21). The 2B5 antibodies were also able to substantially reverse edema formation in a rat adjuvant-induced arthritis model (21). Remarkably, the efficacy of 2B5 in these inflammatory models was indistinguishable from that of indomethacin, a potent NSAID. In the most recent study, 2B5 was shown to be as efficacious as the COX-2 selective inhibitor, SC-58635, in a carrageenan-induced hyperalgesia model in rat (22). It is clear from these as well as previous studies that blockade of EP subtype receptor(s) could conceivably be as efficacious as NSAIDs in the treatment of inflammatory diseases without any of the undesirable side-effects associated with them.

Gastric Antisecretory and Cytoprotective Agents - PGs, especially PGE_2, are known to have mucosal protective effects and act through a number of different mechanisms

(23). Drugs such as misoprostol **6** (Cytotec™), Arthrotec™ (diclofenac + misoprostol) and enprostil **7** have been marketed for the prevention of NSAID-induced gastric lesions (24). Although these drugs suppress acid secretion, their role as cytoprotective agents is critical for therapeutic utility. This is substantiated by studies in which a potent antisecretory drug, ranitidine, failed to prevent NSAID-induced gastric ulcers (25). The major side-effects of drugs such as misoprostol are diarrhea and uterine contractions mediated through one or more of the EP receptor subtypes (26).



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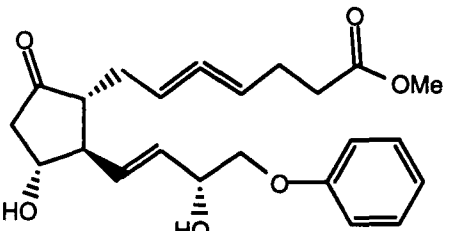

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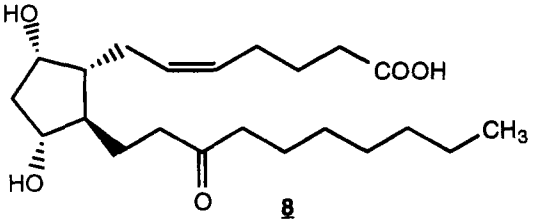
Activation of the EP_3 receptor has been proposed as the receptor responsible for the antisecretory and cytoprotective effects as well as the uterine contractions, while the EP_1 receptor has been postulated as the one responsible for causing diarrhea (26). Misoprostol acts as a prodrug from which the α-methyl ester must be hydrolyzed to the free acid form by esterase activity (27). The prodrug is selective for the EP_3 receptor with a Ki of 319 nM as measured by a radioligand binding assay using membranes prepared from HEK 293 EBNA cells stably expressing one of the eight prostanoid receptors (28). Enprostil, while more potent at the EP_3 receptor with a Ki of 12 nM also shows activity at the EP_1 and FP receptors with Ki's of 82 nM and 88 nM, respectively (28). This would suggest that the EP_3 receptor plays an important role in gastric cytoprotection, however, it is unclear as to the role the EP_1 receptor plays vis-à-vis gastric acid secretion and diarrhea.

Recently, a COX-2 selective inhibitor, L-745,337, has been shown to be effective in a carrageenan-induced hyperalgesia model in rats without causing any gastric ulceration at doses up to 100 times those required to illicit the antinociceptive effects (29). Results from clinical trials with COX-2 selective inhibitors suggest that this may be the case for humans as well obviating the need for prescribing additional gastric cytoprotective agents in the future (30).

Anti-glaucoma Agents - Isopropyl unoprostone (UF-021) **8** and latanoprost **9** (Xalatan™) were recently launched as an anti-glaucoma agents. They reduce



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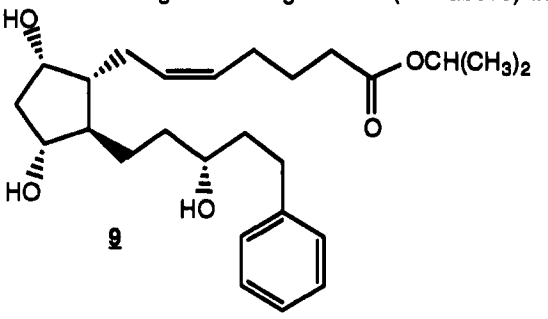
intraocular pressure (IOP) by increasing uveoscleral outflow (31). Both are potent agonists of the FP receptor but unlike $PGF_{2\alpha}$ or other naturally occurring prostanoids they cause very little ocular irritation or hyperemia (31). This is likely due to the fact

that these $PGF_{2\alpha}$ analogues are as potent as the parent compound but show increased receptor selectivity. This is apparent from radioligand binding studies (see above) in which the active drug, latanoprost-free acid, shows potent and selective binding at the FP receptor with a Ki of 2.8 nM but relatively little affinity for any of the other prostanoid receptors (28). On the other hand, the prodrug, latanoprost (isopropyl ester form), is 200-fold less potent at the FP receptor (Ki = 555 nM) (28).



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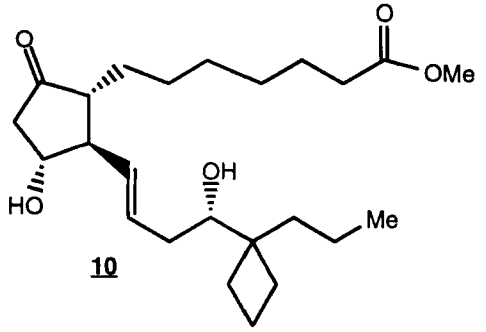
Therefore, a number of effects on human eyes attributed to $PGF_{2\alpha}$, such as an initial short ocular hypertensive response (32), hyperemia, ocular irritation, pain and headache (33) but not to latanoprost are most likely due to its ability to activate additional prostanoid receptors. Two possible candidates include the EP_1 and EP_3 receptors (28). An additional complication which arose while testing various agents was the fact that the IOP response was different in rabbits compared with primates due to receptor as well as mechanism based differences (31).

Anti-asthma Agents - PGE_2 has been shown to induce bronchodilation in man along with significant side-effects of coughing and retrosternal soreness (34). It has also been shown to markedly inhibit the early and late response to allergen (35). The principal mechanism postulated for this inhibition of the late response is thought to involve the inhibition of mediator release from inflammatory cells rather than any dilator effect (35). Therefore, the development of a potent long-lasting PGE_2 agonist without the afore-mentioned side-effects would be desirable. The first compound to be evaluated in man was butaprost **10** which acted as a bronchodilator when administered as an aerosolin. It was not as potent as PGE_2 but neither was it as potent at causing coughing and retrosternal soreness. Butaprost-free acid, the active compound, is very selective for the EP_2 receptor with a Ki of 91 nM, although close to 20-fold less potent than PGE_2 (Ki = 4.9 nM) (28). A compound from a series of thiazole derivatives, under investigation as an anti-asthma agent, is also a potent agonist of the EP_2 receptor (36). Whether or not the EP_2 receptor is the pertinent target has not yet been established. However, development of potent selective EP_2 agonists may clarify this issue.



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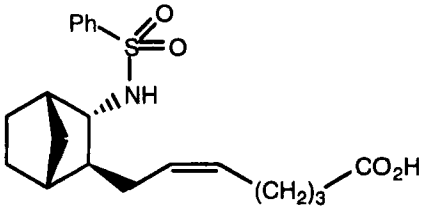

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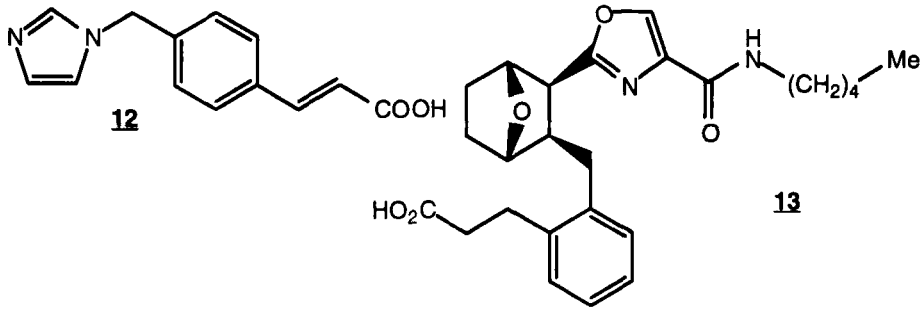
The other important prostanoid agonist in terms of respiratory effects is U46619, a TxA_2 mimetic, which is a potent bronchoconstrictor. It has been suggested that antagonism of the TP receptor might provide valuable therapy for certain airway diseases including asthma (37). Studies with TP receptor antagonists have been

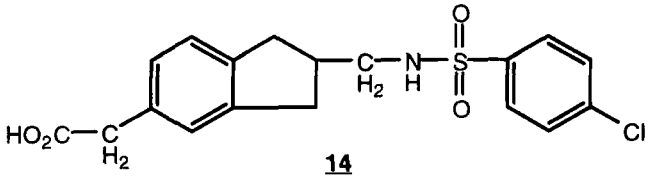
somewhat disappointing (37), however, compounds from this class continue to be of interest. For example, the compound domitroban calcium hydrate (S1452) **11**, which is the (R)-form of the TP receptor antagonist S-145, is under development as an antiallergic and is in phase II clinical trials for allergic rhinitis (38). Although the compound has anti-thrombotic effects it is not being developed for cardiovascular indications. The TxA_2 synthase inhibitor, ozagrel, developed as an anti-thrombotic agent (see below), has been launched as the hydrochloride monohydrate salt of ozagrel (OKY-046) as an oral anti-asthmatic agent (39). It is also in phase II clinical trials for dry cough (40).

Anti-thrombotic Agents - The central role of TxA_2 as an activator of platelets has prompted the development of either TP receptor antagonists, TxA_2 synthase inhibitors or dual antagonist/synthase inhibitors as potent and selective anti-thrombotic agents for a number of cardiovascular related indications; unfortunately mostly without success. Only one compound has thus far made it to market, ozagrel **12**, a TxA_2 synthase inhibitor which was launched for the treatment of cerebral vasospasm following subarachnoid hemorrhage and subsequently for acute cerebral thrombosis, but thus far only in Japan (40).



12 **13**

In terms of TP receptor antagonists still under development, ifetroban (BMS-180291) **13** is currently furthest along (phase II clinical trials) for use as an anti-thrombotic and anti-ischemic agent (41). It is orally active and has been shown to inhibit platelet aggregation and attenuate platelet shape change in healthy males (42). Orally active TP receptor antagonists in phase I include Z-335 **14** for the treatment and prophylaxis of chronic arterial occlusion and LCB-2853 **15** as an anti-thrombotic agent. The latter compound was shown to be vasoprotective in a number of animal models (43).



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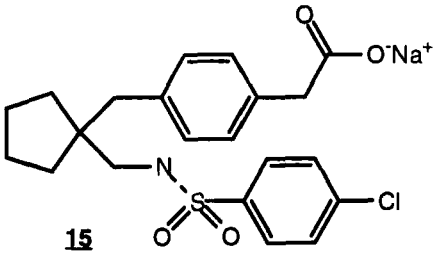
Lastly, terbogrel **16**, also in phase II clinical trials (44), is a dual TP receptor antagonist (K_i = 4 nM) and TxA_2 synthase inhibitor (IC_{50} = 11 nM) being developed as an anti-thrombotic agent (45). Diseases in which TxA_2 is specifically overproduced and/or are insensitive to aspirin such as aspirin-insensitive thrombosis (46) would perhaps benefit from TP selective antagonism (47).



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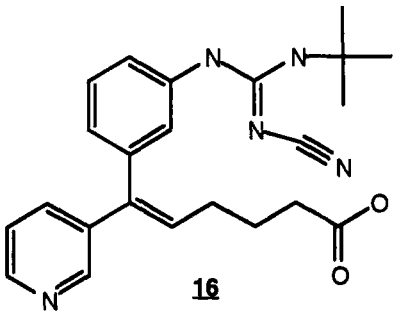

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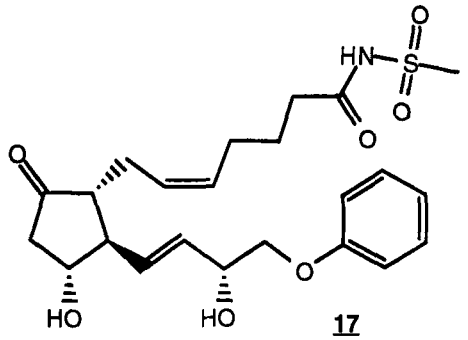
Abortifacients - PGE_2 and $PGF_{2\alpha}$ are thought to be the principal prostanoids involved in myometrial contractions, cervical ripening and initiation of parturition in humans (48). Three drugs have been launched as abortifacients: sulprostone **17**, gemeprost **18** and carboprost trometamol **19** (see Table 1) although none of them have been marketed in the USA. The first two compounds are EP receptor agonists with the third being an FP receptor agonist. Development of compounds having relative tissue selectivity resulted in the synthesis of sulprostone which retained uterine stimulation but had less gastrointestinal effects and little if any blood pressor or bronchial effects (49). In the radioligand binding study, sulprostone shows selectivity for the EP_3 receptor being equipotent to PGE_2 although it is only 10-fold and approximately 60-fold less potent at the EP_1 and FP receptors, respectively (28).



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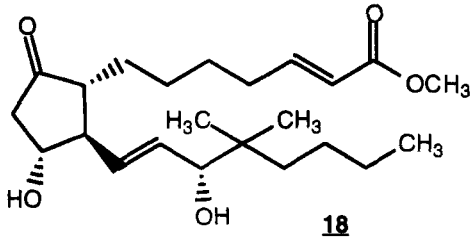

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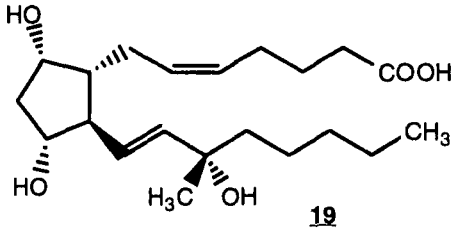

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Another therapeutic area in which a prostanoid receptor antagonist may find utility is preterm labor since it has been known for some time that COX inhibitors, such as indomethacin, can block uterine contractions as well as parturition in humans (50). Administration of indomethacin for preterm labor, however, is problematic due to neonatal complications (51). In this regard a selective prostanoid receptor antagonist may be preferred. The smooth muscle contractile EP_3 and FP prostanoid receptors which are highly expressed in the pregnant human uterus thus could provide a more appropriate therapeutic target for the management of preterm labor (52).

Prostanoid Receptor Knockout Mouse Studies - To date IP, EP_4 and FP prostanoid receptor knockout studies have been reported. Disruption of the IP receptor gene did

not affect heart rate, blood pressure or bleeding time under basal conditions, however, susceptibility to thrombosis increased. Inflammatory and nociceptive responses in IP-deficient mice were reduced and similar to indomethacin treated wild-type mice in a carrageenan-induced paw edema model and acetic acid-induced writhing test, respectively (53). The most striking phenotype in the FP receptor knockout mice was the lack of parturition. The proposed mechanism involves the failure of the corpus lutea to cease progesterone production and that the FP receptor is required for luteolysis which does not occur in FP-deficient mice (54). How this mechanism is related to human parturition remains to be examined. In EP_4 knockout mice of interest was the fact that the ductus arteriosus failed to close after birth resulting in death within 48 hours (55).

Future Outlook - A great deal of effort has gone into the development of prostanoid drugs, a number of which have been launched for various therapeutic indications. Almost all of this was accomplished prior to the cloning of any of the eight prostanoid receptors. Assays then available made it difficult to develop potent, and more importantly, selective compounds, with the exceptions being FP selective agonists for lowering IOP and TP receptor antagonists, although in the latter case thus far without therapeutic success. Cloning of the receptors has opened up a new chapter in prostanoid biology and may lead to the development of useful therapeutic agents in a number of disease categories. Information from knockout mouse studies, tissue and cell localization studies as well as gene regulatory studies will shed important light on the role prostanoid receptors play in normal physiology and pathophysiology. Assays utilizing heterologously expressed prostanoid receptors can now be used to develop potent and selective agonists and antagonists. Finally, testing of these compounds in appropriate animal models will determine their potential therapeutic applicability.

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SECTION V. TOPICS IN BIOLOGY

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Chapter 23. Kinases in Pro-Inflammatory Signal Transduction Pathways: New Opportunities for Drug Discovery

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Introduction - The two pro-inflammatory cytokines tumor necrosis factor alpha (TNF) and interleukin-1-beta (IL-1) are intimately associated with the pathology of inflammation. They are released by fibroblasts and macrophages and act on endothelial and immune cells to initiate and amplify an acute inflammatory response through orchestration of gene expression (1-4). In chronic inflammatory diseases, TNF and IL-1 appear to be involved in the maintenance and perpetuation of the inflammatory state. The two cytokines induce numerous cellular genes that encode a wide array of proteins including cell-adhesion molecules, cytokines, growth factors, chemokines and oxidative stress-related enzymes, which all contribute to the ensuing inflammatory reaction (5-9). Regulation of expression of this diverse set of genes is mediated by the transcription factor Nuclear Factor kappa B (NF-kB) (10,11) a tightly regulated protein complex that is normally maintained in an inactive state in the cytoplasm, and is activated by TNF and IL-1 to migrate into the nucleus and initiate transcription of pro-inflammatory genes (12,13). With the recent discovery of several serine/threonine-specific protein kinases involved in NF-kB activation, the key enzymatic elements of the signal transduction pathways initiated by TNF and IL-1 are now identified (reviewed in 14-17). The stage is set to evaluate and validate these kinases and other regulatory proteins in the pro-inflammatory signaling pathway as therapeutic targets for the discovery of novel anti-inflammatory agents.

TRANSCRIPTION FACTOR NF-kB

NF-kB is a dimeric DNA-binding protein that binds to cognate motifs in enhancer and promoter regions of genes to activate transcription. Originally discovered as a key regulator of the immunoglobulin k light chain gene, NF-kB is a family of proteins that have conserved DNA binding and dimerization domains (10,18). Over the past ten years, high affinity DNA-binding motifs for NF-kB have been found in more than 50 genes that are induced upon inflammatory stimuli. In most cases, mutational inactivation of NF-kB binding motifs abolishes gene induction by TNF, IL-1, and other stimuli that include bacterial lipopolysaccharide, phorbol esters and radiation. NF-kB frequently synergizes with other transcription factors in gene induction. Examples include Activator Protein-1 (AP-1), members of the Signal Transducer and Activator of Transcription (STAT) family, Enhancer Binding Protein (C/EBP) factors and Sp1 (10,11). The finding that NF-kB is a target for the immunosuppressive action of glucocorticoid receptor (GR) agonists highlights its pivotal role in orchestrating a pro-inflammatory response (reviewed in 9). Hormone-activated GR binds directly to NF-kB and attenuates its strong transcription activating potential, providing at least partial rationale for the anti-inflammatory activity of glucocorticoids. This suggests that NF-kB and upstream signaling components that control its activation are valid targets for the discovery of novel anti-inflammatory drugs. The activity of AP-1 is also suppressed by GR, a dimer of Jun and Fos/Fra proteins frequently involved in pro-inflammatory gene expression. In cases where NF-kB synergizes with AP-1 or other transcription factors

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to regulate pro-inflammatory gene expression, mutational analysis has demonstrated that the NF-kB sites are essential for gene induction (11). Such observations provide the rationale that blocking the action of NF-kB alone would be sufficient to inhibit pro-inflammatory gene expression.

The activity of NF-kB is regulated by a family of inhibitory proteins, called inhibitors of NF-kB (IkBs) (12,13). In unstimulated cells, IkB proteins are firmly bound to the NF-kB dimer in the cytoplasm. In this complex, NF-kB is unable to enter the nucleus or to bind to DNA. Stimulation of cells with TNF or IL-1 or a number of other agents causes proteolytic degradation of IkB and allows the released active NF-kB transcription factor to enter the nucleus and initiate gene expression. The cytokine-triggered degradation of IkB requires phosphorylation of two serine residues in the N-terminal portion of the proteins. However, phosphorylation alone is insufficient to release IkB from NF-kB; it but only provides a recognition signal for an E3 ligase that conjugates ubiquitin moieties on to IkB, allowing degradation by the 26S proteasome (19). Proteasome inhibitors have been shown to suppress NF-kB activation and may be expected to exhibit anti-inflammatory activity. However, the proteasome is probably not an attractive anti-inflammatory drug target because it regulates the turnover of many important cellular proteins. Several molecules inhibiting NF-kB activation have been described and the mode of action of anti-inflammatory agents, e.g. salicylates, have in part been attributed to inhibitory effects on NF-kB activation, emphasizing the importance of this pathway in inflammation (9).

An important role for NF-kB in immune regulation is also suggested by the phenotypes observed in knockout mice lacking various DNA binding subunits of NF-kB (19, 20). Mice lacking p50, p52, c-Rel or RelB have various kinds of immunodeficiencies and marked defects in lymph node architecture depending on which subunit is absent. Knockout of p65/RelA, which is the major transcriptionally active subunit of cytokine-induced NF-kB, surprisingly results in an embryonic lethal phenotype. The lack of viable p65-deficient mice makes it difficult to evaluate the role of this subunit in inflammation and immune modulation in adult animals. Some immune cells, such as B-lymphocytes, have constitutively active NF-kB and it is therefore difficult to extrapolate the consequence of inhibiting NF-kB activation from the phenotype of knockout mice lacking NF-kB subunits. Knocking out the gene for a subunit would be expected to affect both constitutive and inducible NF-kB activity, whereas an inhibitor that only prevents activation of NF-kB may not affect the constitutively active NF-kB present in some cell types.

TNF-R1 SIGNALING

Early events in TNF-R1 signaling - Biochemical and genetic approaches using yeast two-hybrid screens were key to the identification of novel proteins which transmit the signals from activated membrane-bound TNF receptors towards the NF-kB-IkB complex in the cytoplasm. Of the two receptors for TNF, TNF-R1(p55) and TNF-R2(p75), receptor subtype 1 is the major signaling receptor for the pro-inflammatory actions of TNF. It initiates at least three distinct signal transduction pathways leading to NF-kB activation, apoptosis, and Jun-N-terminal kinase (JNK) and AP-1 activation (21-23). The cytoplasmic region of TNF-R1 has a protein-protein interaction motif, termed the death domain, which engages in homotypic and heterotypic associations and appears to be a key signaling motif of the receptor (24). Binding of TNF to the extracellular domain results in receptor trimerization and association of the intracellular death domains. The aggregated death domains provide a scaffold for the assembly of a receptor-associated signaling complex of three components: TNF receptor-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP). (25-27). A fourth protein, Fas-associated death

domain protein (FADD/MORT-1) has been shown to be part of the TNF-R1 complex in co-immunoprecipitation studies with overexpressed proteins. FADD may link TNF-R1 to the caspase cascade triggering apoptosis (reviewed in 28,29). TRADD associates with activated TNF-R1 through its death domain and recruits TRAF2 and RIP into the receptor complex. TRAF2 and RIP also have affinity for one another ensuring the formation of a tight complex. This receptor-bound signaling complex then activates a kinase cascade leading to IkB phosphorylation. TRADD primarily serves an adapter role in the complex and overexpression of TRADD initiates both the cell death and NF-kB activation pathways, mimicking TNF-R1 activation (25, 30). Although TRAF2 and RIP have clearly been shown to be components of TNF-R1 signaling complex as discussed below, it is unclear how these components activate downstream signaling events.

The TRAF proteins - The TRAF proteins currently constitute a family of six protein members that have a conserved domain of approximately 230 amino acids at the carboxyl terminus, termed the TRAF domain, and an amino terminal half that has a zinc finger motif (31-39). In addition, all TRAF proteins other than TRAF-1 have an additional N-terminal RING finger sequence. TRAF2, TRAF5 and TRAF6 activate NF-kB when overexpressed and the amino-terminal RING finger motif is required for this activity. The TRAF proteins self-associate and interact with one another via the TRAF domain but it is not known if the proteins exist as homomeric or heteromeric complexes in cells. TRAF proteins associate with several different receptors belonging to the TNF receptor superfamily including TNF-R1, TNF-R2, low affinity NGF receptor (p75), lymphotoxin-beta receptor, CD27, CD30, CD40 and 4-1BB, and are believed to be involved in signaling by these receptors (40). The conserved TRAF domain recognizes the sequence motif PXQXT that is present in several TRAF-binding proteins (41,42). However, the molecular basis for the specific binding of TRAFs to individual receptors is not known and the TRAF recognition site on some receptors has been mapped to sequences other than the PXQXT motif (43,44). TRADD and RIP also bind TRAFs and a mutational analysis of TRAF2 has shown that these proteins also bind within the TRAF domain (45).

Overexpression of TRAF2 activates NF-kB and a TRAF2 mutant lacking the RING finger is dominant-negative and prevents NF-kB induction by TNF (26,32). While such transfection experiments with cultured cells suggested a critical role for TRAF2 in NF-kB activation by TNF, studies with cells from TRAF2-deficient mice or mice expressing a dominant negative form of TRAF2 revealed a near normal activation of NF-kB by TNF (46,47). The signaling defects in these cells were limited to an absence of TNF-induced JNK activation and sensitization to TNF-induced cell death. Hence TRAF2 may not be involved in NF-kB activation by TNF-R1, but rather induction of the JNK pathway and regulation of apoptosis. It is possible that other TRAF proteins like TRAF5 are involved in NF-kB activation by TNF and that TRAF2 is involved in NF-kB activation by other receptors. The observed effects of TRAF2 mutants on TNF signaling in transfection experiments could have been caused by the absence of compensatory TRAF5 in the cell lines used or indirectly caused by formation of non-functional mutant TRAF2/TRAF5 complexes in the transfected cells.

The role of RIP in TNF signaling - RIP is a serine/threonine kinase and is the only component in the TNF-R1 signaling complex with enzymatic activity (27,48). In addition to a kinase domain, RIP has a carboxyl terminal death domain that allows binding to TRADD. RIP, like TRADD, is recruited to TNF-R1 within minutes of addition of TNF, suggesting that RIP is important for signaling. However, no physiological substrates have been identified for RIP kinase nor is the significance of its autokinase activity known. The kinase activity and even the entire kinase domain is dispensable for apoptosis, NF-kB and JNK activation (27,49). A screen for cells defective in NF-kB

activation in response to TNF yielded a cell-line that lacks RIP, indicating an essential role for this protein in signaling (50). TNF signaling could be restored in this cell line with a RIP mutant lacking kinase activity, and a region between the kinase and death domains of RIP was required for restoring function. These results suggest that RIP's role in NF-kB activation by TNF may be restricted to that of an adapter and kinase activity may be irrelevant for the NF-kB pathway. It is possible that transfection studies with overexpressed proteins may have obscured the role of RIP's kinase activity in NF-kB activation by TNF or that there is another TNF-mediated pathway requiring RIP kinase function.

IL-1 RECEPTOR SIGNALING

Early events in IL-1 receptor signaling - In contrast to TNF which signals through a single polypeptide chain, TNF-R1, IL-1 signals through a heterodimer of IL-1 receptor (IL-1R) and IL-1 receptor accessory protein (IL-1RAcP) (51-53). Although the receptors for TNF and IL-1 have no sequence similarity, they each recruit three distinct but functionally analogous proteins as an early signaling step for NF-kB activation. The components in IL-1R signaling that are analogous to the TNF-R1 signaling components TRADD, TRAF2 and RIP are MyD88, TRAF6 and IRAK, respectively (37, 51-55). MyD88 is an adapter that can bind both the receptor complex and IRAK, and recruits IRAK to the IL-1 receptor complex within minutes after IL-1 stimulation. IRAK is a serine/threonine kinase that is rapidly autophosphorylated on receptor activation and IRAK associates with TRAF6 to activate the NF-kB pathway. Overexpression of MyD88 or TRAF6, leads to NF-kB activation, and truncated forms of the proteins can be generated that are transdominant-negative for NF-kB activation. Studies with dominant-negative TRAF2 and TRAF6 mutants indicate that TRAF6 is the TRAF family member specifically engaged by the IL-1 receptor complex. This makes the IL-1 receptor the only non-TNF receptor superfamily member so far employing a TRAF protein.

The role of IRAK in IL-1 signaling - IRAK was discovered as a kinase that associates with the IL-1 receptor complex within seconds of stimulation (57). IRAK binds to MyD88 to enter the receptor signaling complex, and undergoes extensive autophosphorylation. It then probably dissociates from the receptor to activate downstream signaling molecules along with TRAF6, and is subsequently degraded by the proteasome (37, 54-58). IRAK autophosphorylation was induced in T cells by stimulation with interferon-gamma inducing factor (IGIF/IL-18), an IL-1 related cytokine (59). IRAK may therefore be involved in signaling by several cytokines. IRAK-2, a homolog of IRAK identified by search of sequence databases (54), associates with both IL-1 receptor chains, MyD88 and TRAF6, when overexpressed. Both IRAK and IRAK-2 activate NF-kB upon overexpression and truncated mutant forms of IRAK and IRAK-2 specifically block IL-1 induced NF-kB activation, suggesting a role for the proteins in IL-1 signaling (54,55). However, no kinase activity has been demonstrated for recombinant IRAK-2 and it appears to lack some amino acid residues that are invariant in all known kinase domain sequences, supporting that IRAK-2 may lack kinase function. The dominant negative effect of IRAK-2 on IL-1 induced NF-kB activation may therefore result from sequestration of other signaling molecules that associate with the protein. Although IRAK autophosphorylation is detected in IL-1-stimulated cells, no other substrates have been found for IRAK and the role of its kinase activity in NF-kB activation is unknown. Thus it is still unclear how these proteins activate downstream signaling molecules. The role of IRAK and IRAK-2 in IL-1 signaling may be clarified when mice lacking the respective genes are available.

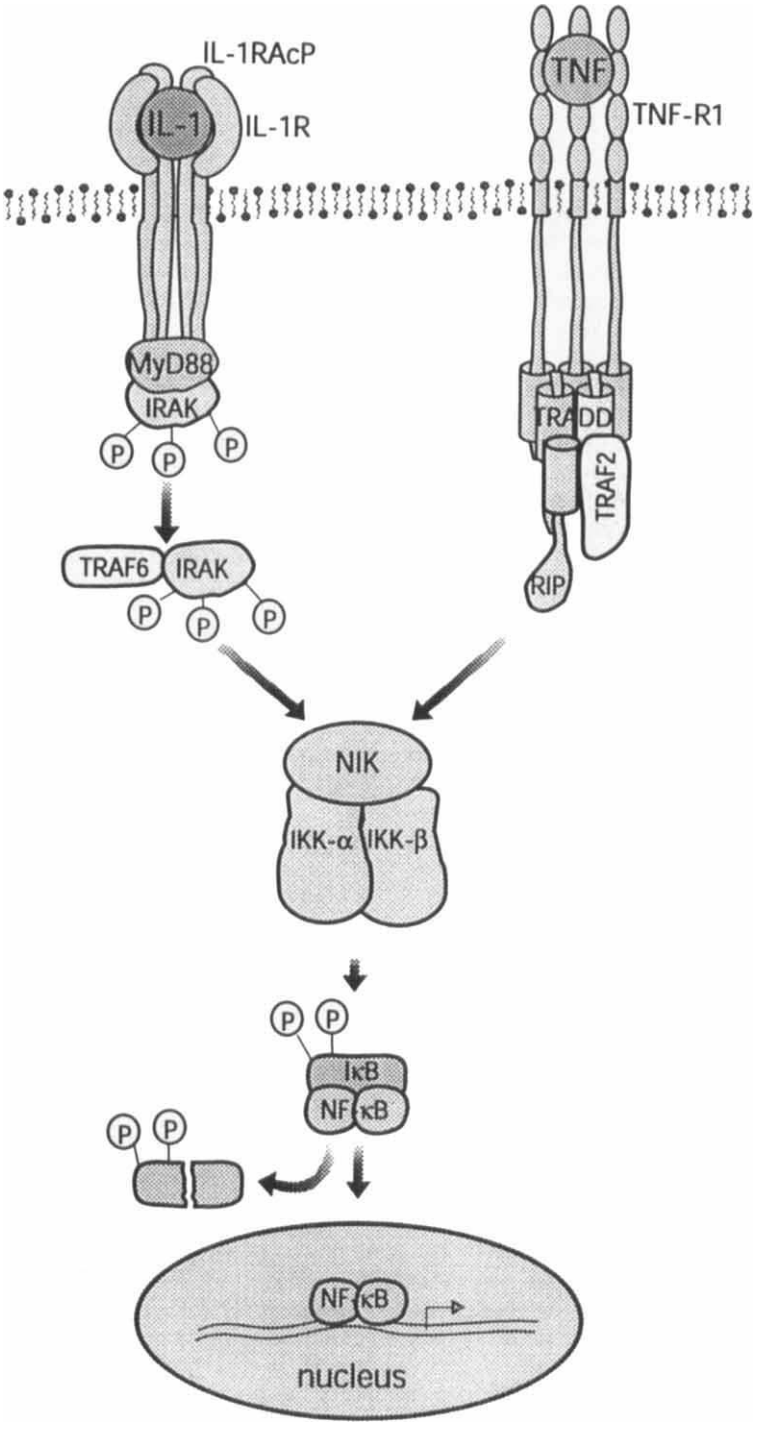

Figure 1. Signal transduction pathway for activation of NF-kB by TNF and IL-1.

KINASES IN NF-kB ACTIVATION

NF-kB-inducing kinase (NIK) - Although the role of TRAF2 in NF-kB activation is unclear, its use in a yeast two-hybrid screen has led to the discovery of a new kinase downstream of the receptor signaling complexes that controls NF-kB activation by TNF and IL-1 receptors. The clone identified encodes a protein closely related to mitogen-activated protein kinase kinase kinase (MAP3 kinase) and was termed NIK (60). NIK associates through its carboxyl-terminal domain with the TRAF domain of TRAF2 but can also associate with TRAF1, 3, 5 and 6, when overexpressed in cells (60,61). It remains to be established which TRAF proteins associate with NIK under physiological conditions and if this association is triggered by cytokines.

NIK activates NF-kB when overexpressed and a kinase inactive mutant or the TRAF-interacting carboxyl terminus of NIK behaves as a dominant-negative mutant blocking NF-kB activation by TNF, IL-1, TRADD, RIP and TRAF2 (60). From these experiments, NIK appears to be the convergent point for TNF and IL-1 mediated NF-kB activation and is the first common downstream target of both cytokine-receptor signaling complexes. NIK is not involved in JNK activation by TNF, and JNK signaling proceeds through the stress-activated MAP kinase pathway (61). How the TNF and IL-1 signaling complexes activate NIK is currently unknown as is the nature of the activating stimulus delivered by the TRAF proteins to NIK. NIK does not directly phosphorylate IkB, suggesting that it may be a kinase controlling the IkB kinase.

IkB Kinase Complex - A key event in NF-kB activation is the inducible phosphorylation of IkB at two serine residues in the amino terminus of the protein (19). Although several kinases had been shown previously to phosphorylate IkB in vivo, none modified the protein at the regulatory serine residues. Two related kinases have now been identified that are part of a multiprotein complex phosphorylating IkB at the regulatory sites (14-16). The first report of a kinase phosphorylating the critical serine residues 32 and 36 in IkB-alpha indicated that this activity was part of a large 700 kDa complex and that, surprisingly, phosphorylation required ubiquitin and ubiquitination enzymes (62). While the purified and molecularly cloned kinases have now been shown to be part of a complex, the function of ubiquitination remains unclear.

IKK-alpha (IKK–α) and IKK-beta (IKK–β) - Two distinct approaches, one relying on protein purification and the other on yeast two-hybrid screening, have identified the same two related polypeptides, termed IKK–α or IKK-1 and IKK–β or IKK-2, as having the requisite IkB-specific kinase activity. In one approach, a cytokine-inducible IkB kinase was purified from cells stimulated with TNF (63-65). The activity resided in a complex of >500 kd. Two polypeptides in this complex were sequenced, their cDNAs cloned, and shown to be IkB kinases. One of them, IKK–α, corresponds to a previously cloned serine/threonine kinase called CHUK (66); the other, IKK–β, is 52% identical to IKK–α. The two proteins have an amino-terminal kinase domain and two putative protein-protein interaction motifs, a leucine zipper and a helix-loop-helix domain in the carboxyl terminal half. Both polypeptides phosphorylate IkB at the requisite serine residues, their kinase activity is induced within five minutes of cellular stimulation by TNF, IL-1 or phorbol ester and activate NF-kB upon overexpression, thus satisfying several criteria for *bona fide* IkB kinases regulating activation of NF-kB.

A connection between NIK and the IKKs was unraveled when IKK–α was independently isolated as an NIK-interacting protein in a yeast two-hybrid screen (67). NIK has been found to associate tightly with IKK–α and IKK–β and NIK associates independently with both the amino and carboxyl half of the two IKKs. NIK enhances the kinase activity of IKK and its capacity to activate NF-kB. NIK thus appears to be a

direct regulator of IKKs and the link between the receptor signaling complexes and the IkB kinases. It remains to be determined, whether NIK is the sole integrator of all NF-kB-inducing signals or if other NF-kB-inducing stimuli activate the IKKs through other NIK-like kinases.

NF-kB Activation by IKKs - IKK proteins form homo- and heterodimes through their leucine repeat region, with the heterodimer being the preferred species (63-68). The helix-loop-helix domain is not required for association but IKK $-\alpha$ or IKK $-\beta$ lacking this region has reduced kinase activity, suggesting a regulatory role for this domain. Several proteins in addition to IKK $-\alpha$ and IKK $-\beta$ copurify with the IkB kinase complex, some of which may interact with the helix loop helix domain of the IKKs (64). The presence of the NF-kB subunit p65/RelA and IkB-beta in the IKK complex may simply reflect the binding of the cytoplasmic NF-kB---IkB substrate to IKKs. The functional role of additional proteins detected in the IKK complex, including MEKK-1, a phosphotyrosine protein and one cross-reacting with an antibody to a MAP kinase phosphatase, is unclear. NIK is also very likely a component of the IKK complex, since studies reconstructed with transfected NIK and IKK $-\alpha$ and IKK $-\beta$ genes suggests that three kinases can form a trimeric complex in cells (68).

The individual contribution of each IkB kinase to the overall activity of the IkB kinase complex is not known. The two ubiquitously expressed IKKs, which prefer to heterodimerize, may be entirely redundant in function. However, the kinase domains of IKK $-\alpha$ and IKK $-\beta$ show only 64% identity, raising the possibility that they may have different substrate specificities, making the two enzymes functionally unique. The evidence available so far suggests that the kinases do not behave identically. Experiments with kinase-inactive point mutants of IKK $-\alpha$ and IKK $-\beta$, which are expected to function as dominant-negative inhibitors of NF-kB activation, have yielded conflicting results about their relative functional importance (64-68). Some studies have found that transfection of a kinase-inactive mutant of either IKK $-\alpha$ or IKK $-\beta$ prevents cytokine-induced NF-kB activation, whereas others have found a dominant-negative effect with only IKK $-\beta$. The differences may be related to the observation that the two kinases associate in cells and that the relative amounts of the endogenous kinases affect the phenotype observed for the overexpressed mutant. IKK $-\alpha$ and IKK $-\beta$ also differ in their potential to activate NF-kB when overexpressed and show different preferences for phosphorylation of one of the two serine residues in various IkB proteins. Analysis of cells with genetically inactivated IKK $-\alpha$ and IKK $-\beta$ genes may eventually reveal the contribution of each kinase to NF-kB activation and phosphorylation of various IkB isoforms.

Regulation of IKKs - The rapid and transient activation of IkB kinases by cytokines indicates that the IKK enzymes are tightly regulated in cells. Both kinases have a potential activation loop sequence within the kinase domain. For IKK $-\beta$, changing two serines within the putative activation loop (Ser 177 and Ser 181) to glutamate, a phosphoserine-mimetic residue, generates a hyperactive kinase that constitutively activates NF-kB in a transfection assay (64). Mutation of the same serines to alanines inactivates the protein and makes it a dominant-negative for NF-kB activation. Thus IKK $-\beta$ appears to be regulated by phosphorylation in a similar fashion as MAP kinases. The kinase phosphorylating IKK $-\beta$ has not been identified, although a likely candidate is NIK. The kinase activity of NIK, however, has not been reported to be induced by TNF or IL-1 and how the phosphorylation of IKK $-\beta$ is regulated by these cytokines is an area of active investigation.

The various proteins that have been identified as components in TNF and IL-1

signaling pathways leading to NF-kB activation fall into two classes. One class contains adapter proteins that provide protein-protein interaction motifs, such as death and TRAF domains for the assembly of cytokine-inducible signaling complexes. The other class contains enzymes with serine/threonine-specific protein kinase domains. RIP and IRAK may fall into either class or exert a dual function. A small molecule inhibitor of the first class would have to disrupt what is likely to be a strong protein-protein interaction. While high-throughput screens that monitor such protein-protein interactions are feasible, the discovery of potent small molecule non-peptide inhibitors (less than 500 daltons) by random screening seems rather unlikely, since mutagenesis studies suggest that the interaction interfaces extend over five amino acid residues. An alternate approach for such targets may be to determine the structure of the protein interface by a combination of structural and mutagenesis studies and then use this information to design peptide or peptidomimetics to disrupt the interaction. A new application of NMR, termed SAR by NMR, uses the NMR structures of protein domains complexed with low-affinity (Kd ~ mM) ligands obtained from random screening to guide the synthetic optimization toward higher affinity ligands. (69,70). Peptide leads can be found by screening peptide libraries displayed on phage particles (71-75). However, to be effective in blocking signal transduction, such peptide inhibitors would have to permeate the cell membrane and disrupt protein-protein interaction. An additional limitation of such an approach is the challenge of converting a peptide lead into a non-peptidic small molecule that will be an orally active therapeutic agent.

Kinases, by contrast, use ATP as a substrate and are found to have binding pockets for adenosine analogs. Random high throughput screening of chemical libraries has produced many potent small molecule inhibitors of kinases (76-81). A major concern about the therapeutic utility of kinase inhibitors is their selectivity. An inhibitor would be exposed to hundreds of kinases inside the cell and selectivity of an inhibitor for a particular kinase may be critical especially for chronic diseases. Most known kinase inhibitors compete for binding of ATP in a region of the enzyme that is relatively conserved among kinases. The increasing number of reports of ATP competitive inhibitors that are selective among distantly related kinases has allayed some early concerns about the near impossibility of designing inhibitors with any degree of selectivity. Now that there are multiple kinase inhibitors in various stages of development, information on the clinical utility of kinase inhibitors should be available in the next few years.

CONCLUSION - Future research should confirm NIK and IKKs as new anti-inflammatory drug targets. The data presently available from cell culture experiments establish NIK and IKK as essential participants in TNF and IL-1 signaling. Further validation of IKKs comes from the fact that they phosphorylate IkB proteins, an event known to be essential for NF-kB activation. However, there are concerns regarding their relative redundancy. The two kinases may have partially overlapping functions, and efficacy in animal models of disease may be achievable only with inhibitors of both enzymes. The discovery of selective inhibitors to these targets should help resolve these questions. Similarly, the identification of specific RIP and IRAK inhibitors may further elucidate the role of their kinase activities in TNF and IL-1 signaling, respectively.

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Chapter 24. Macrophage Migration Inhibitory Factor (MIF): A Critical Upstream Regulator of Acute and Chronic Inflammatory Responses

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Introduction - The biological response to infection and tissue invasion is extraordinarily complex, involving numerous interactions between the host and the invading stimulus (1). Cytokines function in this process by recruiting and activating the critical effector cells that serve to eliminate the source of invasion and preserve host integrity. Glucocorticoid hormones produced by the adrenal glands are important endogenous modulators of inflammation (2-4). When released as part of a systemic stress response, glucocorticoids will effect the course and magnitude of the ensuing outcome (5,6). Baseline glucocorticoid levels also appear to control the "set-point" of the inflammatory reaction (5-8). The central role of glucocorticoids in host immunity can best be appreciated by the extreme compromise exhibited by animals that have had their pituitary or adrenal glands removed. For instance, hypophysectomized rodents without a source of adrenocorticotrophic hormone (ACTH) are extremely sensitive to bacterial infection and endotoxic shock. These mice readily succumb to nanogram quantities of endotoxin (lipopolysaccharide), whereas up to 1000-fold higher doses are required to achieve comparable lethality in normal animals (9,10). Pharmacologically, glucocorticoids remain the most powerful anti-inflammatory and immunosuppressive substances that have been identified and their widespread clinical utility is limited only by dose-dependent side effects leading to diabetes, hypertension, osteoporosis, cataracts, and growth arrest (5).

In contrast to other hormonal networks which regulate carbohydrate, electrolyte, or blood pressure homeostasis, no systemic mediator(s) have been identified that could counter-regulate the powerful effects of glucocorticoids on the immune system. This situation prompted exploratory studies to find potentially new mediators that might be released systemically and that could counter the suppressive effects of glucocorticoids on inflammation and immunity. As part of this experimental program, an apparently novel 12.5 kD protein was cloned that was identified to be secreted by the corticotrophic, pituitary cell line AtT-20 (11). Upon sequencing, it was found that this pituitary peptide shared very high homology with a recently cloned human "cytokine" called macrophage migration inhibitory factor (MIF) (12). MIF was first described in the early 1960s as a product of activated lymphocytes that could inhibit the random movement or migration of cultured macrophages/monocytes (13). In 1966, Bloom and David independently characterized this activity to be a soluble factor produced by activated T lymphocytes (14,15). This engendered significant interest as MIF became one of the first soluble, non-immunoglobulin factors that was amenable to study in vitro. Nearly 25 years passed before MIF was cloned however, and this was due largely to difficulties in identifying an abundant cellular or tissue source of the protein for amino acid sequence determination.

Structure of the MIF Gene - The mouse and human MIF genes are highly homologous in their coding regions and display a similar intron/exon organization (16,17). With the exception of a 27% sequence homology with the enzyme L-dopachrome tautomerase (described below), neither mouse nor human MIF display significant homologies with any other known DNA sequences. The mouse MIF gene is located on chromosome 10

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and maps to a position coincident with several recessive mutations, such as the grey lethal (gl), mocha (mh), and grizzled (gr) (17). At the present time however, there is no indication that mutations of the MIF gene result in a known mouse defect or disease. The gene for human MIF has been localized to chromosome 19 (18).

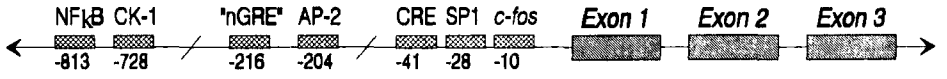

Fig 1. Schematic illustration of the mouse MIF gene (adapted from ref 17).

The promoter region of the mouse MIF gene contained several consensus sequences that may be involved in transcription factor binding (17)(Fig 1). These enhancer/regulatory binding domains, many of which exist in the promoter for human MIF as well (16), include a sequence motif implicated in the basal expression of the proto-oncogene c-fos, an Sp-1 site, a c-AMP responsive element (CRE), an AP-2 site, and a possible "negative" glucocorticoid responsive element (nGRE), all located in close proximity to the RNA transcription start site. A cytokine-1 (CK-1) site and a nuclear factor-κB (NF-κB) site also were identified further upstream on the minus DNA strand. Functional promoter studies are needed to determine the role of these and other promoter elements in the expression of the MIF gene. Nevertheless, it appears that the MIF promoter contains regulatory sequences that are characteristic of both cytokine (NF-κB) and endocrine hormone (CRE, nGRE) genes (16,17). Northern blotting analysis of mouse tissue showed that the MIF gene is transcribed as a single, 0.6 kB mRNA species. A survey of MIF expression in different organs indicated the presence of constitutive MIF mRNA in many tissues that were examined. Whether this represents the expression of MIF by parenchymal cells, resident tissue macrophages, or circulating blood cells remains to be established. However, recent immunohistochemistry studies indicate that MIF also is present in many non-immune cells, where it may play an autocrine or paracrine role in cell regulation (19,20).

Structure of the MIF protein - The recombinant human and mouse MIF proteins (rhuMIF and rmuMIF, respectively) are 90% identical throughout their respective 115 amino acid sequences and have been compared biochemically to native muMIF prepared from liver, a rich source of MIF (21). On SDS-PAGE gels, all three proteins exhibit relative molecular weights (M_r) between 12,000 and 13,000, which are in good agreement with the values predicted by their amino acid sequences. MIF is not significantly glycosylated nor have other posttranslational modifications been found. X-ray analysis of crystals formed from either wild type or selenomethionine-MIF indicated that MIF has a trimeric structure (21-27). MIF is an α/β structure that forms a homotrimer with dimensions of approximately 35 Å x 50 Å x 50 Å. Six α-helices surround three β-sheets that completely wrap around to form a barrel containing a solvent-accessible channel (25). Four of these β strands comprise a β sheet while the two remaining β strands interact with the β sheets of the two adjacent monomers to connect the subunits. Together, the three β sheets form a barrel that surrounds a solvent channel along a 3-fold axis of symmetry. The amphipathic helices of each monomer are packed along the exterior surfaces of the β sheet of their respective monomers. The channel formed by the three monomers is lined with hydrophilic residues and resembles two large funnels that empty toward the center of the trimer. Although several negatively charged residues are present at each end of the channel, the overall electrostatic potential of the channel is positive. This suggests the channel may interact with negatively charged molecules.

Fig. 2. Three-dimensional structure of human MIF, viewed down its three-fold axis of symmetry (modified from ref. 25).

MIF displays a weak primary (amino acid) sequence homology (27%) with the enzyme D-dopachrome tautomerase, and significant three-dimensional structural (but no primary sequence) homology with two recently crystallized bacterial enzymes: 4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (28). Interestingly, while CHMI exists as a trimer similar to MIF, 4-OT is organized as a hexamer. The 4-OT monomer is 62 amino acids long and dimerizes to form a structure that is topologically similar to the MIF monomer, consisting of two α-helices lying on top of a four stranded β-sheet. The pseudo 2-fold axis of symmetry that exists in the MIF monomer is thereby replaced by an exact 2-fold axis in 4-OT. Accordingly, three 4-OT dimers arrange to form a hexamer with an overall 3-dimensional structure that is highly homologous to that of MIF. Of note, each of these bacterial proteins catalyzes an isomerization reaction and the recent observation that MIF also has enzymatic activity (see below) points to the intriguing possibility that an enzymatic reaction may underlie some of the immunological properties of MIF.

The relative small diameter of the MIF channel (5 Å) suggested that it would allow small molecules to move through the trimer (25). MIF has been reported to bind glutathione (23,24,29) and it catalyzes the tautomerization of D-dopachrome (30). The presence of negative charges on these small molecules suggests that they may interact with positive charges in the channel. However, NMR studies have shown that MIF binds glutathione very weakly, and comparisons between the amino acid sequences of MIF and D-dopachrome tautomerase revealed that none of the identical shared residues occupies a position that lines the MIF channel (7). Thus, it appears that the MIF channel is probably not the site of either glutathione or D-dopachrome binding or of D-dopachrome catalysis.

Enzymatic Activity of MIF - An investigation of melanin biosynthesis led to the discovery of an enzymatic activity that converted a non-physiological isomer of 2-carboxy-2,3-dihydroindole-5,6-quinone (D-dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (31). The protein responsible for this activity was purified, partially sequenced, and found to be identical to MIF, and the presence of this activity was subsequently confirmed in recombinant MIF (32). MIF catalyzed the tautomerization of D-dopachrome less efficiently than D-dopachrome tautomerase, but preferred esterified versions of the D-dopachrome, suggesting that a related derivative may be the physiological substrate of MIF. The 4-OT-specific substrate, 2-hydroxymuconate, is a weak substrate for MIF (35). Despite little amino acid sequence homology, MIF, 4-OT, and CHMI have a common N-terminal proline

residue that is important for the catalytic activity of these molecules (33,34). Deletion or substitution of the proline residue of MIF, or moving it interior to the N-terminus by adding more N-terminal amino acids, rendered MIF devoid of tautomerase activity and indicated that the presence and position of the N-terminal proline are critical for MIF activity. Substitution of two other basic residues of MIF, His41 and His63, did not affect enzyme activity. Deletion of 5 or 11 residues from its C-terminus removes it ability to form trimers and renders it catalytically inactive.

MIF mutants lacking enzymatic activity continue to demonstrate full activity in glucocorticoid override assays, indicating that the tautomerase activity is not required for this immune function of MIF (35). However, this does not rule out a role for the tautomerase activity in some other capacity of immune signaling. The widespread expression of MIF in the CNS, its capacity to utilize esterified versions of either stereo isomer of dopachrome, and the abundance of dopachrome-like peptides in the CNS, suggest that MIF enzymatic activity may be involved in modifying the bioactivity of dopachrome-containing neuropeptides (35). Identification of a physiological substrate for MIF should reveal a novel role for MIF in addition to its important regulatory function in the immune system.

The MIF Receptor - Very few studies of interactions between MIF and its cognate cellular receptor have been reported and a macrophage surface receptor protein for MIF has yet to be identified. Early work using unpurified preparations of MIF activity indicated that carbohydrate residues on the surface of monocytic cells were important for mediating migration inhibition by MIF (36,37). Subsequent studies indicated that the counteraction of MIF activity found associated with carbohydrate factors, either added or cleaved from the cell surface, was likely caused by a de novo activation of macrophage migration through a MIF-independent pathway rather than by disrupting the functional interaction between MIF and its receptor (38).

An anti-monocyte monoclonal antibody, Mo3e, blocks the inhibition of human monocyte migration by purified MIF (39). The Mo3e antibody recognized protease-sensitive antigens on two proteins with estimated molecular weights of 80 kD and 50 kD. Expression of the 50 kD protein increased following exposure to MDP, PMA, and LPS, consistent with the behavior of proteins involved in macrophage activation or a putative MIF receptor. The functions and identities of these proteins remain to be addressed, and it is premature to conclude that these proteins are related to a MIF receptor.

MIF-Binding Proteins - Sarcolectin is a growth-promoting lectin that antagonizes the biological effects of α/β interferon, such as induction of 2-5A synthetase (40). During a search for proteins that bind to sarcolectin, MIF was unambiguously identified as a protein that bound sarcolectin specifically and with high affinity (K_D values of 2.1 and 113 nM) (41). Sarcolectin also was shown to block MIF activity in a macrophage migration assay (42). Further study of sarcolectin revealed that it is identical to human serum albumin (43). The role of the sarcolectin/albumin-MIF interaction has not been defined but the binding of free MIF by a serum protein may serve to modulate widespread MIF activity and limits its action to the desired afflicted tissue.

MIF-Related Proteins - Glycosylation inhibiting factor (GIF) is a 13 kD protein that contains an amino acid sequence identical to MIF except for a single substitution: MIF contains a serine and GIF contains an asparagine at position 106 (44). GIF inhibits the glycosylation of IgE-binding factors, which can then suppress IgE synthesis in their unglycosylated form. This activity of GIF evidently contributes to the formation of antigen-specific suppressor T cells. Although MIF and GIF are virtually identical in sequence and in structure, they possess quite distinct bioactivities. Whereas MIF acts

on macrophages and T-cells in its native state, GIF requires posttranslational modification for its bioactivity (45). This uncharacterized posttranslational modification is only known to be achieved by suppressor T-cells in vivo. However, bioactivity of recombinant GIF can be recovered by chemical modification of its Cys60 residue (45). While the MIF receptor is doubtless present on macrophages, high affinity GIF binding was detectable on helper T hybridomas, helper T-cell clones, and natural killer cells, but was notably absent from macrophages (46). Despite the structural similarities between MIF and GIF, the marked differences in their respective bioactivities illustrate that seemingly minor alterations can have profound physiological consequences.

CELLULAR AND TISSUE SOURCES OF MIF

Anterior Pituitary Gland - The anterior pituitary gland is an abundant source of MIF protein. Immunocytochemical and ELISA analyses have shown that MIF accounts for approximately 0.05% of the total pituitary protein content (11). By comparison, the values for the "classical" pituitary hormones ACTH and prolactin are 0.2% and 0.08%, respectively (47,48). In mice, MIF was detected within the corticotrophic cells of the anterior pituitary gland, where it resides in secretory granules (49). We have also observed that cultured, primary pituitary cells release significant quantities of MIF after stimulation with corticotrophin-releasing factor (CRF) (49). Studies in rodents indicate that MIF is an integral component of the systemic stress response (50). Following the intraperitoneal administration of LPS in mice, the total pituitary content of MIF falls by 38%, as assessed by enumeration of immunogold-labeled MIF granules (11,49). There is a corresponding increase in the circulating concentrations of MIF protein and, at the same time, an increase in the pituitary content of MIF mRNA (11). Activation of the hypothalamic-pituitary-adrenal (HPA) axis by handling stress also results in a time-dependent elevation in plasma MIF. Serum MIF increases in parallel with ACTH and glucocorticoid levels, and reaches levels of ≈50 ng/ml within 3 hours (50). These data indicate that pituitary MIF is secreted into the circulation during inflammation (endotoxemia) and stress, and provide support for the concept that MIF is an important component of the hypothalamic-pituitary-axis response.

Monocytes/Macrophages - For almost 30 years, MIF "activity" was considered a product of the activated T lymphocyte. Yet in addition to the pituitary, the monocyte/macrophage is also an important source of MIF in vivo (51). Unlike other cytokines, high baseline levels of MIF mRNA and MIF protein are present, pre-formed, in unstimulated macrophages. Macrophage MIF is released upon stimulation with pro-inflammatory stimuli, such as gram negative endotoxin (LPS), the gram positive exotoxins toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxin A, hemozoin (malaria pigment), and the cytokines TNFα and IFNγ (51-53). Of note, the LPS concentration required to up-regulate MIF expression is at least two orders of magnitude lower than that needed to induce the expression of TNFα, suggesting that the MIF release response is extremely sensitive to pro-inflammatory stimuli. The secretion of MIF follows a bell-shaped dose-response curve in response to increasing concentrations of bacterial toxins, decreasing at high concentrations of LPS or TSST-1. The fact that MIF production is turned off at high concentrations of bacterial products may be an important protective mechanism of the host to prevent the detrimental effect of excessive amounts of MIF release (with its attendant glucocorticoid overriding activity). MIF was also found to induce TNFα secretion by macrophages and to synergize with IFNγ for nitric oxide production (20,51). These data indicate that MIF may act in concert with TNFα and IFNγ to amplify the pro-inflammatory responses of macrophages.

Like the corticotrophic cells of the anterior pituitary gland, the macrophage also contains pre-formed stores of MIF (51). This may account in part for the rapid

accumulation of MIF protein in media upon LPS stimulation in vitro (51). By immunofluorescence and confocal microscopy, these pre-formed stores of MIF protein appear to be concentrated within intracytoplasmic granules. The macrophage MIF release response to LPS decreases by approximately 1000-fold in the absence of serum (11), suggesting that serum factors such as lipopolysaccharide-binding protein (LBP) may play a role in MIF release, potentially via activation of a CD14-dependent pathway (54,55). In the absence of serum, CD14-negative cell lines such as the AtT-20 pitiutary cell line will release MIF in response to LPS. However, this requires microgram concentrations of LPS in medium. Glucocorticoids exert a large part of their anti-inflammatory effects by inhibiting pro-inflammatory cytokine production. Thus, a pivotal turn in the investigation of MIF biology came by the finding that glucocorticoids induce, rather than inhibit, MIF secretion (50). The glucocorticoid-induced production of MIF from macrophages also exhibits a classic bell-shaped dose response curve. Peak MIF release occurred at low concentrations of dexamethasone and decreased at higher dexamethasone levels. Similar results were obtained upon stimulation of macrophages with cortisol, the primary human glucocorticoid hormone, and with mouse or human T lymphocytes as the cellular source of MIF (50,56).

The observation that a potent anti-inflammatory agent such as cortisol could induce the macrophage and the T cell to secrete a "pro-inflammatory" cytokine was at first puzzling. This apparent paradox was resolved when MIF, added together with dexamethasone to monocytes, could overcome, in a dose-dependent fashion, glucocorticoid inhibition of TNFα, IL-1β, IL-6 and IL-8 secretion by monocytes (50). This effect was demonstrable for primary monocytes, as well as for thioglycollate-elicited, mouse peritoneal exudate cells, and the RAW 264.7 macrophage cell line. Moreover, the ability of rMIF to "overcome" glucocorticoid inhibition of cytokine secretion also was observed in alveolar macrophages obtained from patients with the acute respiratory distress syndrome (ARDS) and thus activated in vivo, in the absence of exogenous LPS (57).

One would predict from this study that endogenously-released MIF could act in an autocrine fashion to overcome glucocorticoid inhibition of TNFα production. This was verified experimentally by studying the effect of neutralizing anti-MIF antibodies on human monocyte cultures that had been treated with both LPS and dexamethasone. When human monocytes were stimulated with LPS in the presence of dexamethasone and control IgG, TNFα production was inhibited by only 9%, compared to 32% for monocytes with LPS in the presence of dexamethasone and a neutralizing anti-MIF IgG (50). Thus, anti-MIF was found to potentiate markedly the inhibitory effect of dexamethasone on monocyte TNFα secretion and the balance between the glucocorticoid and MIF concentrations was the main determinant of cytokine production in this in vitro model system.

T-Cells – Historically, MIF was considered a T cell cytokine, and the macrophage, its main cellular target. Accordingly, very little is known about the effect of MIF on T cell or B cell responses. The presence of pre-formed MIF in resting T cells was recently demonstrated (51,56). Mouse spleen and human peripheral blood T cells, purified free of monocytes/macrophages, express MIF mRNA and release MIF protein when stimulated with mitogens, anti-CD3 antibody, or specific antigen. Recently, in a classic model of the delayed-type hypersensitivity (DTH) response, the macrophage rather than the T lymphocyte is the predominant source of MIF protein and mRNA expression but anti-MIF has a blocking effect (50). The staphylococcal superantigen TSST-1 was also found to be a very potent inducer of MIF secretion from mouse splenic T cells (52).

Subsequent experiments established that MIF is a critical component in the pathway leading to T cell activation. Anti-MIF antibody was shown to inhibit T cell proliferation and IL-2 production in vitro, and to suppress antigen-driven T cell activation and antibody production in vivo (56). This was consistent with the observation that T_H2 lymphocyte cells produced the highest amounts of MIF. As in macrophages, glucocorticoids in low concentrations induced MIF secretion by T cells, and MIF was found to override, in a dose-dependent fashion, the glucocorticoid-mediated suppression of T cell proliferation induced by anti-CD3 antibodies (56) and associated restoration of IL-2 and IFNγ production. The interaction between MIF and glucocorticoids appears to be tightly regulated, as reflected by the precise stoichiometry of MIF and glucocorticoid required to regulate cellular effects (56). However, no direct, high-affinity binding of glucocorticoid to MIF has been demonstrated.

ROLE OF MIF IN DISEASE

Endotoxemia and Sepsis - Like TNFα, IL-1β and IFNγ, MIF is an important mediator of the acute inflammatory response. When co-injected with LPS into mice, MIF potentiates lethal endotoxemia (11). Conversely, neutralizing polyclonal or monoclonal anti-MIF antibodies fully protect mice from endotoxic shock (11). Yet, in contrast to TNFα and IL-1β which have direct, pro-inflammatory actions on immune, endothelial, and other cell types, the effects of rMIF in vivo appears to result primarily from its regulatory effects on target immune cells (11,50). Thus, in the absence of an inflammatory stimulus (such as LPS), rMIF by itself does not cause hypotension or significant toxicity when injected systemically in rodents (11,50). The observation that anterior pituitary cells release MIF adds further to the potential level of host controls that could trigger MIF release during infection or tissue invasion (11,51). Recent studies have also established that MIF is a pivotal mediator of shock induced by gram-positive organisms, and neutralizing polyclonal or monoclonal anti-MIF antibodies will fully protect mice from lethal TSST-1 administration (52).

Acute Respiratory Distress Syndrome (ARDS) - In established ARDS, significantly elevated levels of the mediators IL-1, TNFα, and several chemokines are found in the alveolar airspaces and can be sampled in bronchoalveolar lavage (BAL) fluid (58). When we assayed lavage fluid from patients with ARDS for immunoreactive MIF, we have found that these patients had significantly elevated levels of alveolar MIF (57). By immunohistochemical analysis, significant quantities of MIF protein were also found to be associated with macrophages and with Type II alveolar epithelial cells (57).

Inflammatory monocytes prepared from the BAL fluid of patients with ARDS spontaneously release cytokines such as TNFα and IL-1. Not surprisingly, these "in situ" activated cells also secreted MIF. The addition of a neutralizing anti-MIF antibody to these BAL monocyte cultures significantly attenuated the production of TNFα and chemokines (57). Conversely, the addition of rMIF to BAL monocyte cultures increased further the spontaneous production of these cytokines by 80-100%. Of pivotal importance however, was the finding that MIF could override glucocorticoid effects in these ARDS-derived, in situ-activated cells. Thus, while the addition of glucocorticoids markedly inhibited cytokine production from BAL monocytes, rMIF was found to override, in a concentration-dependent manner, the immunosuppressive effects of glucocorticoids on these cells.

Glomerulonephritis - During the development of anti-GBM glomerulonephritis, there is a marked upregulation of MIF expression by intrinsic kidney cells including endothelium and glomerular and tubular epithelial cells, as well as by infiltrating

macrophages and T cells (59). To address the role of MIF as an immunomodulator in this autoimmune disease, rats were treated with a neutralizing anti-MIF mAb (IIID9) or an isotype control mAb from the time of anti-GBM administration until sacrifice 14 days later. As expected, the control antibody-treated group developed severe proteinuria and renal function impairment with marked histological damage due to leukocyte infiltration and glomerular injury. By contrast, the anti-MIF treated rats showed substantially lessened proteinuria, significant preservation of renal function, reduced histological damage including glomerular crescent formation, and decreased renal leukocyte infiltration and activation (all $p<0.001$ compared to controls). Inhibition of renal disease by anti-MIF mAb was attributed to preventing the marked upregulation of IL-1, the leukocyte adhesion molecules ICAM-1 and VCAM-1, and the inducible isoform of NO synthase. The inhibition of progressive renal injury was mirrored by the complete suppression of the skin DTH response to the challenge antigen, rabbit IgG (60). These data, which were the first extensive characterization of the protective effects of anti-MIF, provide strong evidence that MIF is an important regulator of immune-mediated kidney diseases.

Rheumatoid Arthritis - In the rodent, MIF protein is present constitutively within the epithelial cells lining the synovium. In joints obtained from mice with collagen-induced arthritis, MIF expression increased in the synovial lining cells and was readily detected in association with infiltrating macrophages and to a lesser extent, T cells, as well as in endothelial cells abutting areas of inflammation (61). We have also studied the effect of neutralizing anti-MIF antibodies in collagen Type II-induced arthritis in mice of the B10.QxDBA/1 strain. Treatment with anti-MIF mAb versus control IgG1 led to a significant lower frequency and delay in the onset of arthritic pathology. This was associated with a lower level of serum IgG2a directed against collagen Type II (61).

In a model of rat adjuvant arthritis, MIF expression was detectable by immunohistochemical methods 4 days after injection (6 days prior to onset of the clinical symptoms) (62). In addition, MIF co-localized with ED-1+ cells, indicating that the presence of MIF in macrophages in adjuvant arthritis synovium may be important in the initiation of the disease. Monoclonal anti-MIF treatment dose dependently inhibited the clinical score of arthritis, paw swelling, and synovial leukocyte lavage numbers ($p<0.001$) and also resulted in reduced synovial macrophage and T-cell accumulation (62).

We have measured MIF levels by ELISA in synovial fluids obtained from the knee joints of human subjects suffering from two common forms of arthritis. In patients with rheumatoid arthritis, an inflammatory and oftentimes rapidly destructive form of arthritis, mean MIF levels were 9.4±4.7 ng/ml (n=20). In a group with osteoarthritis (generally a non-inflammatory and less destructive disease), mean MIF levels were 3.7±2.3 ng/ml (n=10, $P<0.005$) (63). Synovial tissue cells isolated from arthritic joint tissue (obtained peri-operatively) secrete constitutively in culture several cytokines, including TNFα, IL-1, and chemokines (IL-8, MCP-1, MIPs). Incubating these cells with a neutralizing anti-MIF antibody was found to inhibit by 60% spontaneous TNFα production. These data further affirm the important regulatory role of MIF in the inflammatory and immune processes associated with arthritis. Taken together, these experiments provide important supportive data for the critical role of this mediator in the host immunological response.

Given the critical, upstream role of MIF in regulating inflammatory and immune responses, anti-MIF therapeutics may have potential benefit in sepsis, ARDS, and chronic inflammatory diseases such as glomerulonephritis and rheumatoid arthritis. A multiplicative strategy of inhibition is supported by data from ARDS patients indicating that MIF may regulate TNFα, IL-8 and other important redundant and interacting

cytokines and chemokines involved in the systemic inflammatory response syndrome. Most importantly, anti-MIF therapeutics may ultimately be used in conjunction with, or as a replacement for steroid therapy. In the future, cloning and characterizing the MIF receptor, and understanding its structure, distribution, and signaling properties will lead to an improved understanding of this important pro-inflammatory and glucocorticoid regulating signal paradigm.

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Chapter 25. The Bcl-2 Family: Targets For The Regulation Of Apoptosis

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Introduction - Programmed Cell Death And Its Role In Disease - The development and homeostasis of multicellular organisms involve the complex orchestration of cell division, differentiation and cell death. During development, cell death must occur to eliminate unneeded cells produced in the course of morphogenesis and organogenesis. In the adult, a steady-state number of differentiated cells in each tissue is achieved by regulating the balance between cell proliferation and death. In recent years cell death is recognized as a complex and actively regulated process, termed "programmed cell death" or "apoptosis" (1,2). "Apoptosis" describes a morphologically defined process of cell shrinkage, membrane blebbing and nuclear condensation and is distinct from "necrosis", the cell death induced by severe and abrupt cellular injury that involves cell swelling and lysis.

The genetic components controlling programmed cell death were first identified in the nematode *C. elegans* by H. R. Horvitz, et al. In that organism, three key genes control the cell death pathway (3-5). The genes *ced-3* and *ced-4* are required for programmed cell death to occur whereas *ced-9* inhibits these deaths. Biochemical experiments demonstrated that Ced-4 protein can directly interact with Ced-3 and Ced-9 (6-10). All of these nematode proteins are now known to have mammalian counterparts. Ced-3 is a member of the caspase family of cysteine proteases, that carry out the execution phase of programmed cell death. The first identified member of this family of proteases was interleukin-1-beta converting enzyme (ICE) (11). Apaf-1 has recently been identified as the first mammalian counterpart of Ced-4 (12). Apaf-1 serves as an adapter molecule that binds to the zymogen form of caspase-9 and leads to its activation by proteolytic processing, analogous to the function of Ced-4 that binds and activates the Ced-3 caspase. Ced-9 is homologous to the Bcl-2 family of proteins which is the subject of this review (13).

Insufficient levels of apoptosis as well as excessive levels can cause or contribute to the pathogenesis of many human diseases. For example, cancer can be considered a consequence of the failure to undergo apoptosis. Normal cells are maintained by survival signals. Sensors of cell damage lead to withdrawal of survival signals and subsequent cell death. By contrast, cancer cells can survive in spite of cellular abnormalities, and the requisite survival signals. This is accomplished by upregulation of anti-apoptotic proteins that raise the "apoptotic threshold" (Fig. 1). Autoimmune diseases are characterized by insufficient apoptosis of self reactive T-cells which, under normal circumstances, would be eliminated. Downregulation of the immune response is achieved normally through apoptosis, induced by activation of Fas, a member of the death receptor family of proteins. MRL-lpr mice with a mutation in the Fas receptor die with symptoms of systemic lupus erythematosus (15).

While failure of apoptosis results in cancer and autoimmune diseases, excess apoptosis may result in a number of degenerative diseases. Examples include disorders in hematopoiesis such as chronic neutropenia, aplastic anemia and myelodysplastic syndrome. Apoptotic neurons have been observed in a number of neurodegenerative diseases such as amytrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease and in ischemic stroke (16-20).

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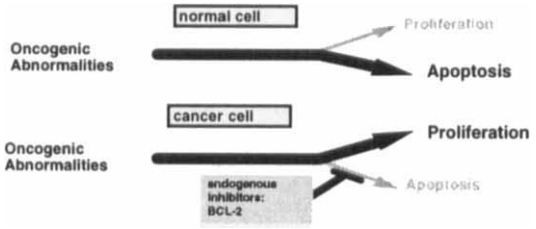

Figure 1: role of apoptosis in cancer cells

Bcl-2 Family Proteins In Cancer - Bcl-2 was isolated in 1985 from the breakpoint of a t(14;18) chromosomal translocation derived from a follicular lymphoma (21-23). Analogous exploitation of cytogenetic abnormalities in tumor cells was used successfully to isolate a number of oncogenes that enhance cell proliferation. However, Bcl-2 contributes to tumorigenesis by promoting survival rather than driving proliferation. The importance of the Bcl-2 oncogene was underscored when it was found to be a structural homologue of the *C. elegans* cell death inhibitor Ced-9. Indeed, Bcl-2 could partially substitute for ced-9 in the living nematode (13,24,25). Further work has now identified a family of Bcl-2 related genes in vertebrates.

In follicular lymphomas, the t(14;18) translocation leads to strong transcriptional activation of Bcl-2 and is the primary event in initiating multistep tumorigenesis. Bcl-2 was upregulated by unknown mechanisms in other human tumor types (Table 1). In prostate cancer, Bcl-2 expression increases with tumor progression from the androgen dependent to androgen independent phenotype. In these tumors Bcl-2 upregulation presumably occurs at a later stage during tumorigenesis. Several studies also suggest association of the Bcl-2 homologue Bcl-xL with cancer (26,27). It was best demonstrated in an elegant study in which Bcl-xL was upregulated during progression of islet beta-cell tumors in transgenic mice (28). In this model, suppression of apoptosis by Bcl-xL occurs during the transition from angiogenic precursor lesions to solid tumors.

Suppression of apoptosis by Bcl-2 family proteins also confers resistance to chemotherapeutic agents. In vitro studies in numerous cancer cell lines demonstrate that overexpression of Bcl-2 or Bcl-xL leads to multidrug resistance against most tested agents (29). Several clinical studies in prostate cancer and chronic lymphocytic leukemia show good correlation of Bcl-2 expression levels in the tumor with drug resistance during therapy (30,31).

Tumor type	Frequency of abnormal Bcl-2 expression	Reference
Non Hodgkin's Lymphoma	40-80%	(32-34)
Prostate (at diagnosis)	20-40%	(31)
Prostate (hormone resistant)	80-100%	(35)
Breast	60-80%	(36-38)
Colorectal	50-70%	(39-41)
Small Cell Lung	60-80%	(42)
Non-small Cell Lung	20-40%	(43,44)

Table 1: Frequency of abnormal Bcl-2 expression in human tumors

Structure Of The Bcl-2 Family Of Proteins - The Bcl-2 family of proteins can be divided into three groups based on sequence homology and biological function (45). Sequence homology analysis further led to the definition of four "Bcl-2 homology domains" (BH 1-4) (Fig. 2).

Fig. 2: Schematic representation of the three subgroups of Bcl-2 family proteins with prototypic family members (TM: transmembrane domain)

The first subgroup of Bcl-2 family proteins contains the members Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 that share homology over all four BH domains and function as inhibitors of apoptosis (46-49). The second subgroup contains Bax, Bak and Bok, and shares homology in domains BH-1-3 but these proteins promote apoptosis in most contexts (50-54). The third group contains Bad, Bid, Bik, Bim and harakiri that share homology only in the BH-3 domain and promote apoptosis (55-59). All members of the first two groups have a C-terminal putative transmembrane domain. In some proteins from the third group, this hydrophobic domain is absent. By immunocytochemistry and cell fractionation methods, Bcl-2 was found to be associated with intracellular membranes in mitochondria, the endoplasmic reticulum and nuclear envelope (45). Bcl-xL and Mcl-1 also have a similar subcellular distribution. Interestingly, recent studies suggest that a significant fraction of Bax protein is cytoplasmic in spite of the presence of a putative transmembrane domain (60). Extensive mutagenesis studies have been performed to elucidate the function of the BH domains (66,67). All four domains were necessary for the survival function of Bcl-2 and Bcl-xL (68). The BH-3 domains of the pro-apoptotic family members Bax and Bak were necessary and sufficient to kill cells (69,70). Homodimers have been demonstrated for Bcl-2 and Bax and heterodimers between various family members have also been observed (61,62). Proteins from the subgroup with homology only in the BH-3 domain are unable to form homodimers or heterodimers within the subgroup, but they dimerize readily with proteins from the other subgroups (57,63-65). BH-1, BH-2 and BH-3 domains all contribute to the ability to form dimers (45).

The NMR and crystal structures of Bcl-xL provided a better understanding of the homology domains and related mutagenesis data (71). Bcl-xL has a core structure of seven helices with an unstructured loop region inserted between the first two helices. The surface of Bcl-xL contains a hydrophobic "pocket" formed with contributions from the BH-1, 2 and 3 homology domains. The NMR structure of a complex of Bcl-xL with a 16-mer peptide derived from the alpha-2 helix of Bak suggests that the alpha-2 helix of one molecule occupies the "pocket" of the dimerizing partner (72). Hydrophobic interactions as well as salt bridges contribute to the binding. It is likely that the structures of Bcl-2 and Bax are very similar given their sequence homology with Bcl-xL. Interestingly, the structure of the Bcl-xL monomer is not consistent with the ability of Bcl-2 and Bax to form homodimers and heterodimers with each other. In Bcl-xL, the hydrophobic face of the amphipathic alpha-2 helix that provides the contact points for binding into the pocket of a partner molecule is not exposed. Thus, a conformational change must occur so that an alpha-2 helix is available for homo- or heterodimer formation. The dimer thus formed will be asymmetrical: one binding partner provides a binding pocket as seen in the crystal structure and the other partner, in a different conformation, provides a short helical binding peptide. Competition studies with BH-3 peptides suggest that homodimers and heterodimers can be formed by this mechanism (73) despite alternative interpretations of yeast two-hybrid binding data (70,74).

Bcl-xL also shows structural homology with the pore forming subunits of diphtheria toxin and bacterial colicins. These proteins, and perhaps Bcl-xL can exist either in a

soluble compact conformation or a membrane inserted conformation, the latter being able to form pores or channels with measurable ion conductance. Bcl-2, Bcl-xL and Bax can form pores and ion flux across membranes has in fact been measured for these proteins (75-77).

POSSIBLE MECHANISMS OF ACTION OF BCL-2 FAMILY MEMBERS

Despite extensive studies of the structure/function relationship of Bcl-2 family proteins and the identification of numerous interacting proteins, the mechanism by which Bcl-2 family proteins regulates apoptosis is not well understood. Several possibilities are discussed below.

Inhibition of Downstream Death Effectors By Direct Interaction - The model derived from studies in nematodes predicts that binding of Ced-4 to the zymogen form of Ced-3 leads to its auto-catalytic processing and activation and that Ced-9 inhibits activation of Ced-3 by binding to Ced-4 (78). In mammalian systems, the Ced-4 homologue Apaf-1 interacts directly with pro-caspase-9 (12). Addition of Apaf-1 to cell extracts in the presence of dATP and cytochrome C leads to activation of caspases (79). Bcl-xL can interact directly with Ced-4 (6,8,9,78). However, evidence for a direct interaction between Bcl-xL and Apaf-1 has not been found. In cells, Bcl-xL can be co-precipitated with pro-caspase-8, suggesting the presence of a bridging molecule (8,80). Collectively, these data led to the concept of an "apoptosome" complex of Apaf-1-like molecules, pro-caspases and Bcl-2 family proteins which controls the activation of capsases. In this model, Bcl-2 family members inhibit cell death by sequestering Apaf-1 and blocking caspase activation.

Death Induction By Bax, and its Sequestration By Bcl-2 – Experiments in yeast and mammalian cells suggest that Bax or other pro-apoptotic family members have an autonomous pro-apoptotic function. Transfection of Bax and Bak can kill the yeasts S. cerevisiae and S. pombe although the genome of S. cerevisiae does not contain any recognizable Bcl-2 homologue that might act as a dimerization partner for Bax (81). Mutants of Bax were created with the goal of distinguishing the dimerization function of Bax from a putative apoptotic function in mammalian cells. Some mutants in the BH-3 domain in Bax retain full pro-apoptotic function but have lost the ability to form dimers with Bcl-2 (82). Such an autonomous function of Bax could possibly be explained by its ability to form ion-conductive pores or by its ability to release cytochrome c from mitochondria. In this model, Bcl-2 is not required to induce death but it would block cell death by binding Bax and inhibiting its autonomous killing capacity.

Pore Formation By Bcl-2 Family Proteins - The structural homology with bacterial toxins predicted that Bcl-xL might also form ion conducting pores created by its central alpha-5 and alpha-6 helices. Bcl-2, Bcl-xL and Bax have been examined for such pore or channel forming properties and they were able to induce measurable ion flux across liposomes and planar lipid bilayers (75-77). Bcl-xL and Bcl-2 exhibit moderate cation selectivity whereas Bax channels are moderately anion selective. Apparently conductance, reflecting channel size, can vary significantly. This is consistent with the progression from channels formed by dimers with a total of four central helices to multi-subunit pores with conductance rates comparable to the mitochondrial permeability transition pore or 'megachannel'. Other indirect evidence also support the notion that Bcl-2 family proteins can form pores: purified Bax can lyse neurons in culture at micromolar concentrations and Bax added to isolated mitochondria leads to the release of cytochrome c (83). The pore-forming ability of Bax may also be responsible for its ability to kill yeast cells and bacteria. Furthermore it has been claimed that early in the apoptotic process the swelling of mitochondria and bursting of the outer mitochondrial membrane can be prevented by Bcl-xL, possibly by forming a

pore that equalizes osmotic pressure (84). Before this hypothesis is accepted, one must determine if Bcl-2 family proteins form pores inside cells at physiological concentration and if the opening and closing of such pores regulate ion flux or the transduction of proteins such as cytochrome c.

Inhibition Of Cytochrome c Release From Mitochondria - Apaf-1 was discovered by the isolation of factors that induce caspase activation in the cytosol. These results provide a model in which caspase-9 is activated by interaction with Apaf-1, and cytochrome c and dATP are required as cofactors (79). Cytochrome c must be released from mitochondria in order to be available for the activation of caspases in the cytosol. Release of cytochrome c from mitochondria is indeed a hallmark of programmed cell death (85). Several studies have shown that Bcl-2 and Bcl-xL can prevent the release of cytochrome c from mitochondria and this maybe the primary mechanism by which Bcl-2 family members block apoptosis (86-89). However, microinjection of Bcl-xL in the cytosol have shown that its action cannot be solely explained by sequestration of cytochrome c (90).

REGULATION OF BCL-2 FUNCTION

Regulation Of Bcl-2 Function By Dimerization - Titration experiments with Bcl-2 and Bax have shown that these proteins form a functionally important network of homo- and heterodimers (56). Cells with high levels of Bcl-2 homodimers are protected from apoptosis induced by growth factor withdrawal. Gradual increase of Bax expression in these cells leads to increasing levels of Bcl-2/Bax heterodimers and Bax homodimers accompanied by gradual loss of protection. However, these experiments could not identify which of the binding partners was the active molecule with the pro-apoptotic or anti-apoptotic effector function and which was the regulator, or whether the heterodimer was in fact the active moiety. Although a clear answer still has not emerged, supportive data have been generated in experiments using the proapoptotic family members Bad and Bid that have homology only in their BH-3 domains and lack the central helices found in pore forming proteins. These molecules, as well as truncated versions of Bax and Bak containing BH-3 domains, are able to induce cell death (45). Such proteins can bind Bcl-2 and Bcl-xL, and these results support the view that Bcl-2 and Bcl-xL are active inhibitors of apoptosis and that the BH-3 containing proteins bind and inactivate them. Additional experiments with Bcl-xL mutants capable of inhibiting apoptosis but unable to bind Bax or Bak further support the view that the death-inhibitory family members have intrinsic anti-apoptotic functions (67). However, other work suggests that the death inducing effect of the BH-3 containing protein Bid correlates with its ability to bind to Bax but not with its ability to bind Bcl-2 or Bcl-xL, supporting a primary role for Bax as an active death inducer (57).

Regulation Of Bcl-2 Function By Phosphorylation - Phosphorylated Bcl-2 has been demonstrated in living cells (91). One phosphorylation site has been mapped to serine-70, located in the loop region between the alpha-1 and alpha-2 helices. Deletion mutants in this loop in Bcl-2 and Bcl-xL are "super active" at least in some experimental contexts (92). It is therefore attractive to hypothesize that modifications of this loop region will have a modulating effect on biological activity. However, existing data do not provide a clear picture of the effect of phosphorylation at this time. On one hand, phosphorylation of Bcl-2 has been associated with enhancement of cell survival upon growth factor withdrawal. Here protein kinase C has been implicated as the responsible kinase (93). On the other hand, phosphorylation of Bcl-2 occurs during apoptosis induced by taxol and other microtubule-targeted chemotherapeutic agents. These data have been used to argue that phosphorylation inactivates Bcl-2, leading to cell death (94).

There is clearer evidence that serine-phosphorylation of Bad leads to inactivation of its pro-apoptotic function (56). Bad exerts its pro-apoptotic function by binding via its BH-3 domain to membrane associated anti-apoptotic family members. When phosphorylated, Bad is sequestered in the cytosol by association with 14-3-3 protein. 14-3-3 is a motif-specific phosphoserine-binding protein with multiple isoforms and has been ascribed many regulatory functions. The serine/threonine kinase Akt-1, also known as protein kinase B (PKB), was recently found to phosphorylate Bad in vitro and in vivo on at least one of the known phosphorylation sites (95-97). As Akt-1 is intimately involved in "survival signaling", for instance through IGF-1 and PDGF receptors, it is possible that phosphorylation of Bad-like molecules by Akt-1 is part of the anti-apoptotic effect of these growth factors.

Other Potential Mechanisms - The search for downstream effectors of Bcl-2 function led to the isolation of numerous Bcl-2 binding proteins. These proteins can either modulate Bcl-2 function or serve as actual downstream effectors of Bcl-2 action. These include calcineurin (98), R-ras (99), raf-1(100), bag-1 (101), p53-BP (102), bap-31 (80), prion protein (103), and nip-1-3 (104). To date, none of these interactions has been definitively identified as essential for the anti-apoptotic function of Bcl-2.

THERAPEUTIC INTERVENTION

Regulators of the Bcl-2 family have potential as novel cancer therapy. However, modulation of Bcl-2 family function can also be used potentially to enhance cell survival in degenerative diseases. Based on the current understanding of the biochemistry of Bcl-2 family members, several technical strategies can be exploited in order to modulate their pro- or anti-apoptotic effects.

Dimerization Blockers - Apoptosis is induced when BH-3 containing proteins bind to the hydrophobic pockets of Bcl-2 and Bcl-xL. Thus, we expect that small molecules that occupy these pockets can be pro-apoptotic agents. Dimerization inhibition assays can be designed to screen for compounds that occupy the pockets since dimerization depends on binding at these sites. BH-3 peptides are the prototype inhibitors, as linear peptides of 15 or more amino acids can completely block dimerization reactions (73). The contact sites of the peptides in the binding pocket on the surface of Bcl-xL are restricted to a small number of known residues. The inhibition constants of such peptides are in the nanomolar range and are only about five-fold weaker than those of their respective full length proteins (72,73). Full length Bcl-2 family proteins show a clear rank order for their preferred dimerization partner, reflected in the relative preference of some BH-3 domain peptides for specific family members (63). This indicates that the binding pockets in different Bcl-2 family members may be distinct.

To screen for dimerization blockers, several types of assays have been established. Dimerization can be measured in living yeast cells in a 2-hybrid format (61). Alternatively, the dimerizations can be recapitulated in vitro using plate-binding assays with either full length proteins or labeled BH-3 domain peptides as "ligands" (73). However, to date, no small molecule blockers of the Bcl-2 family have been reported. One can also envision using dimerization assays to screen for functional enhancers of Bcl-2 activity. For example, if Bcl-2 exists in part as an inactive dimer complexed with Bax, compounds that block Bax binding without inactivating Bcl-2 function, would be expected to enhance cell survival.

Channel Blockers - Assays measuring Bcl-2 family ion channel activity are, in principle, suitable for screening of small molecule libraries. For example, the release of carboxyfluorescein from liposomes has been described (105). Alternatively, ion sensitive dyes can be used to detect ion flux. However, no results from actual drug discovery efforts employing Bcl-2 family members in such assays have been reported

to date. An indirect way to measure potential Bax "pores" is provided by the fission yeast S. pombe. Bax-induced death in these yeast cells is accompanied by cytochrome c release and is inhibitable by Bcl-2 (81,106). Such yeast strains can be used to screen for small molecule inhibitors of this process.

Modulation Of Phosphorylation - Given that the phosphorylation of Bad is a physiologically relevant functional modification, and that the activity of other family members may also be modified in this way, compounds that affect these phosphorylations and dephosphorylations may have therapeutic utility. For example, Akt-1 has been identified as one candidate Bad kinase. Inhibitors of this kinase or other Bad kinases would be expected to promote Bad's pro-apoptotic activity. In a related fashion, inhibitors of a relevant phosphatase would promote the inactivation of Bad, with an expected anti-apoptotic effect. Similarly, inhibitors of the kinases and phosphatases that regulate the phosphorylation state of Bcl-2 may have important functional effects. However, further work will be necessary to characterize the relevant enzymes and the effects of specific phosphorylations.

Gene Therapy, Antisense and Ribozymes - Gene therapy with Bcl-2 family members has been proposed as promoters and inhibitors of apoptosis. Bcl-xS, a splice form of Bcl-xL with pro-apoptotic effects, has been used successfully in an adenovirus delivery vector to kill cancer cells *in vitro* as well as in xenografts in nude mice (107,108). Bcl-2 in a Herpes virus delivery system protects partially from ischemic damage in animal models of stroke (109).

Numerous studies have demonstrated that Bcl-2 antisense oligonucleotides or plasmids can induce apoptosis in tumor cells *in vitro* (110,111). This has been corroborated in mouse models of lymphomas and melanomas (112). One clinical trial with Bcl-2 antisense therapy in patients with non-Hodgkin lymphoma produced preliminary but encouraging results (113). Antisense oligonucleotides to Bax have been shown to promote survival of rat sympathetic neurons in culture (114).

Ribozymes could potentially be used to either enhance or inhibit apoptosis by targeting anti-apoptotic or pro-apoptotic family members respectively. To date, Bcl-2 directed ribozymes have been constructed and used to sensitize hormone-resistant prostate cancer cells to apoptotic agents in vitro (115).

Future Directions - The understanding of the biological function of Bcl-2 family proteins is incomplete. Nevertheless, based on current knowledge, first generation assays have been created to allow identification of molecules that act as antagonists or agonists of members of this family. Progress in assay development will likely follow progress in understanding the biochemical mechanisms by which Bcl-2 family proteins control apoptosis.

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Chapter 26. Chemokine Receptors As HIV Co-Receptors: Targets for Therapeutic Intervention in AIDS

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Introduction - Chemokines are a family of closely-related proteins of about 70-120 amino acids involved in normal leukocyte trafficking and recruitment of cells to sites of inflammation. Following engagement of their receptors, chemokines activate several signal transduction pathways that induce a variety of functional responses including chemotaxis, granule release, superoxide production, integrin upregulation, cellular differentiation and proliferation (for review see 1-3). The number of human chemokines now approaches 50, and the list is likely to continue to grow.

Members of the chemokine family are structurally related, at both the amino acid and three-dimensional levels (4). A shared motif, consisting of conserved cysteine residues that are involved in determining tertiary structure, is a hallmark of this family of proteins and has provided a convenient means of classification. The CXC, or α chemokine class, is characterized by having the first two cysteines separated by one amino acid and act primarily on neutrophils and lymphocytes. In the CC, or β chemokine family, members have the first two cysteines adjacent and tend to act on a variety of leukocytes including T cells, monocytes, macrophages, eosinophils, basophils, natural killer cells, and dendritic cells. The CXC and CC chemokine classes make up the majority of known chemokines. Two additional classes of chemokines have recently been identified. The C class, represented by lymphotactin, is a chemoattractant for lymphocytes (5). The CX_3C class, represented by fractalkine and neurotactin, acts on T lymphocytes and monocytes, and is the only class of chemokines shown to be membrane-bound (6).

One of the major functions of chemokines is to modulate the trafficking of select populations of leukocytes from the vasculature to extravascular sites of action in both normal and disease settings. Much of the early work in this field focused on the role of chemokines in recruitment of leukocytes to sites of inflammatory or allergic diseases. It is now clear that chemokines are also involved in directing immature lymphocytes to sites of maturation, and in determining the character of an immune response to antigen. Thus, chemokines play a major role in crafting the responsiveness of the immune system of an individual. In addition, chemokines have now been implicated as mediators of other functions including angiogenesis and angiostasis, apoptosis, and hematopoiesis.

Interest in chemokines by the HIV community began in 1995, when researchers determined that a combination of the chemokines, MIP-1α, MIP-1β and RANTES, could greatly reduce infection of susceptible cells by macrophage-tropic (M-tropic) forms of HIV (7). The notion that chemokine receptors may function as co-receptors for HIV infection was further supported when an IL-8-like receptor, denoted "fusin" (later renamed CXCR4), was identified via an elegant expression cloning approach as the co-receptor for T cell-tropic (T-tropic) forms of HIV (8). Within weeks, several

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groups simultaneously identified CCR5 as the major co-receptor for infection by M-tropic forms of HIV (9-12). Investigation of CCR5 and CXCR4 as co-receptors has become one of the most intensive areas of research in the chemokine field.

Due to their key roles in recruiting specific populations of cells in inflammation and allergy to sites of disease, and their role as HIV co-receptors, chemokines and chemokine receptors have become a favored target for drug discovery at pharmaceutical companies. Descriptions of monoclonal antibodies, peptides or altered peptides, and small molecules that block chemokine action are beginning to appear in the literature. These compounds are proving to be useful tools for proof-of-concept studies and may lead to effective therapies. Owing to the identification of chemokine receptors as HIV co-receptors, an intensive effort to generate therapeutics to treat AIDS via this mechanism has developed in a very short period of time.

Over 800 papers were published in the chemokine field in the last year alone. As a result, this will not be a comprehensive review, but will instead attempt to highlight some of the major advances in the field. The focus of this review will be on chemokine receptors as HIV co-receptors, and development of chemokine antagonists to treat AIDS. Topics to be reviewed include: 1) characterization of co-receptor usage; 2) genetic susceptibility to HIV infection; 3) characterization of the interactions between HIV gp120 and CCR5 or CXCR4; 4) requirements for signal transduction during HIV infection; 5) temporal and spatial expression of CCR5 and CXCR4; 6) identification of new chemokine receptors as HIV co-receptors; and 7) development of chemokine antagonists as treatments for AIDS. For more comprehensive overviews of the chemokine field, the reader is directed to reviews cited earlier. We apologize to our colleagues whose work has not been cited, but due to space limitations, it was not possible to include all areas of research, or all citations in this very active area.

CHARACTERIZATION OF CO-RECEPTOR USAGE BY HIV

Early work in the field suggested that M-tropic HIV used CCR5 and TCLA (T-cell line adapted), or T-tropic strains of HIV, used CXCR4 as co-receptors. Recent work suggests that co-receptor usage more closely correlates with ability of HIV to form syncytia (13-16). Nonsyncytium-inducing (NSI) strains, found early in infection, are believed to be the more predominantly transmitted species, and are associated with milder disease. NSI strains of HIV use CCR5 and infection can be blocked by β-chemokines. SI (syncytia-inducing) forms of HIV, found later in the disease, are usually associated with more severe disease symptoms. SI forms of HIV use CXCR4 for infection. These reports also showed that the evolution or switch from the NSI to SI forms of HIV in patients correlated with resistance to inhibition by β-chemokines and could be associated with changes in the V3 loop of the HIV gp120-protein (13,17).

GENETIC SUSCEPTIBILITY TO HIV INFECTION

Identification of CCR5 as the major co-receptor for M-tropic HIV led to an interest in determining if there was a genetic basis for susceptibility to HIV infection. Using PCR and high-throughput sequencing, several groups identified a 32 base pair deletion in the CCR5 gene (CCR5delta32) that results in a frameshift mutation, giving rise to a shortened CCR5 protein that is expressed intracellularly (18,19). Additional polymorphisms have been identified in the CCR5 gene, but their role in CCR5 function and HIV susceptibility, if any, remains to be determined (20). $CD4^+$ T cells from delta32 patients are resistant to infection by M-tropic strains of HIV, but can be infected by both T-tropic and dual-tropic HIV. Moreover, several studies indicated that individuals who were homozygous for the delta32 mutation were resistant to HIV infection, despite repeated exposure to the virus (21-23). Individuals who are

heterozygous for the delta32 mutation remain susceptible, but there is evidence for a slower progression to AIDS. No survival advantage was observed in patients whose virus used CXCR4 as the co-receptor. These results identify CCR5 as the co-receptor for infection of primary macrophages and T cells by M-tropic HIV, and the single, most important host factor yet identified for HIV transmission. Other chemokine receptors can be used as co-receptors for in vitro infection by M-tropic HIV (i.e. CCR3, CCR2), however, the importance of these receptors, in in vivo HIV infection and disease progression is not clear. Thus, blockade of CCR5 may prove to be a very effective therapeutic approach. Additionally, individuals homozygous for the delta32 mutation display no remarkable medical history due to lack of CCR5, suggesting that such a therapeutic approach should have few detrimental effects. It should be noted, however, that other host factors are also involved in HIV infection, since 96% of highly-exposed, uninfected individuals are not delta32 homozygotes. These unknown molecules may prove to be equally useful as drug discovery targets for the prevention of AIDS.

The initial work on the delta32 mutation suggested that individuals possessing the homozygous mutation were protected from HIV. There are now several reports indicating that this protection may not be complete. A number of individuals have been identified who are incapable of expressing CCR5, yet still become infected (24-26). The viruses isolated from these individuals were homogenous and contained substitutions in their gp120 proteins commonly found in SI strains of HIV (25). These viruses presumably used CXCR4 to establish the infections. The subjects rapidly lost $CD4^+$ T cells, and progressed more quickly to full-blown AIDS than individuals expressing wild-type CCR5. The increased rate of progression to disease could be the result of decreased immune function due to absence of CCR5, or more likely, due to infection with an SI form of the virus (26). These results suggest that while CCR5 is the major co-receptor involved in HIV transmission and establishment of infection, viruses using CXCR4, or perhaps other as yet unknown co-receptors, can be transmitted in rare cases.

CHARACTERIZATION OF THE INTERACTIONS BETWEEN HIV GP120 AND CCR5 OR CXCR4

There has been an intensive investigation into the regions of gp120 and CCR5 that are responsible for the interaction between HIV virions and the target cell. This interaction appears to be quite complex. The interaction between gp120 and CCR5 involves the V3 loop of the gp120 protein, and this region determines tropism and co-receptor usage (17, 27-29). Taken together with previous findings on interaction of CD4 with gp120, these results have led to a model in which binding of CD4 to gp120 results in a conformational change, revealing the V3 loop of gp120, which can then interact with CCR5 or CXCR4 (Fig. 1).

Other studies have used a variety of model systems to define the regions of CCR5 involved in interaction with both HIV and β-chemokines. Chimeric CCR5 molecules have been created between human and mouse CCR5, since the latter does not function as an HIV co-receptor (30,31). The results suggest that all 3 extracellular domains of CCR5 are important for interaction with gp120, but the relative importance of the domains differ depending on the strain of virus used. The ADA strain of HIV uses a chimera containing just one human extracellular domain, whereas BaL required the presence of 2 human extracellular domains. The dual tropic 89.6 required all 3 human extracellular domains to interact efficiently with gp120.

Similarly, human CCR5/CCR2 chimeras were used to study gp120/CCR5 interactions because HIV uses CCR5, but in general not CCR2, and because human CCR2 and CCR5 are among the most closely related β chemokine receptors (32,33).

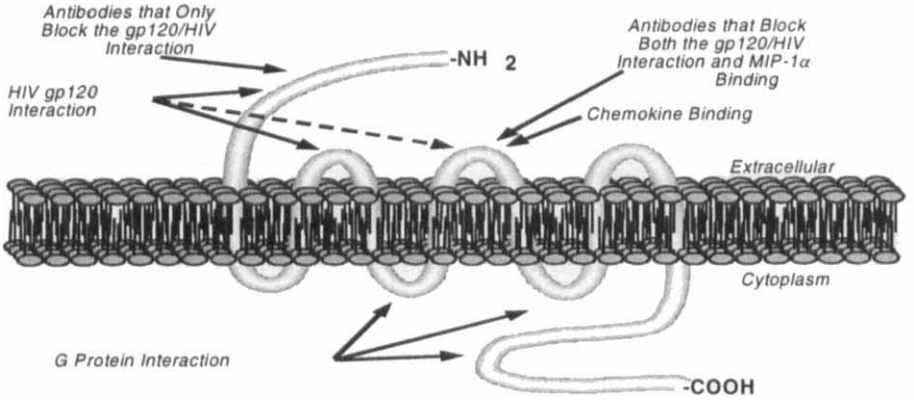


Figure 1 Regions of the CCR5 protein involved in interaction with HIV gp120 and chemokines. Sites indicated as important for HIV interaction are from the chimeric receptor studies of Bieniasz et al. (30), Picard et al (31), Rucker et al. (32), Atchison et al (33), and Farzan et al. (36). Chimeric receptor chemokine binding and activity studies of Samson et al. (34), Atchison et al. (33), indicate the second extracellular loop as being important for chemokine binding and signaling. Antibody binding and inhibition activties from Wu et al (35).

Results from these studies indicated that M-tropic strains required sequences from the N-terminal region, or first extracellular loop, and that the first 20 amino acids of CCR5 were sufficient to support M-tropic gp120 membrane fusion. Conversely, the second extracellular domain of CCR5 is most important for interaction with β-chemokines (34). A monoclonal antibody that binds to the second loop of CCR5 blocks both chemokine and gp120 binding, while antibodies directed to the amino terminal region are unable to block chemokine binding but can block M-tropic gp120 binding (35). Additionally, a tyrosine-rich region close to the CCR5 animo terminus was critical for M-tropic and dual-tropic gp120 interactions and viral entry (36).

The region of CXCR4 necessary for infection of T-tropic and dual tropic viruses was investigated using human/rat CXCR4 chimeras (37,38). All chimeras could be infected by the LAI strain of HIV, which can use both human and rat CXCR4. T-tropic strains that use only the human CXCR4, and dual tropic strains, capable of using either CXCR4 or CCR5, could infect cells only if the chimera contained the human third extracellular domain. An antibody recognizing an epitope in the third extracellular region of CXCR4 confirmed its importance in virus infectivity by T-tropic and dual tropic viruses. A chimera containing the N-terminus of CCR5 on the body of CXCR4 could be used as a co-receptor by M-tropic, T-tropic and dual-tropic viruses, providing support for a model in which the virus evolves throughout the disease process with respect to co-receptor usage (38).

REQUIREMENTS FOR SIGNAL TRANSDUCTION DURING HIV INFECTION

Because chemokine receptors transduce signals upon chemokine engagement, it was of interest to determine if receptor signaling was required for HIV infectivity. Constructs were generated with mutations in several regions of CCR5, including residues in the second intracellular loop ("DRY" box) and carboxy terminus, which are known to be involved in chemokine receptor signaling as measured by calcium flux. These mutants could serve as efficient co-receptors for virus entry comparable to the

wild type receptor even though they were incapable of transducing a functional signal (39-41). A chimera containing the CCR2 N-terminus in place of the CCR5 N-terminus, could signal after binding MIP-1β, but was ineffective as an HIV receptor (39). These results all show that chemokine signaling and HIV co-receptor activities are clearly separable, and that signaling by the receptor is not essential for HIV infectivity. Similar results have been shown for CXCR4 (38).

TEMPORAL AND SPATIAL EXPRESSION OF CCR5 AND CXCR4

To better understand the role of chemokine receptors in HIV infection and transmission, a number of studies have focused on characterization of chemokine receptor expression, and in particular, regulation of expression in different circulating populations of cells. CCR5 was shown to be highly expressed on IL-2 stimulated T cells and also expressed on a subset of memory T cells in the peripheral circulation (42). Conversely, CXCR4 was found predominantly on unactivated naïve T lymphocyte subsets, and levels of this receptor were further increased following activation of T cells via PHA or IL-2 (43).

CCR5 is highly expressed on Th1 cells and nearly absent from Th2 cells (60). T cells from delta32 heterozygotes showed a significant reduction of CCR5 expression on peripheral blood T cells (42). However, there was also considerable variation in levels of CCR5 expression on peripheral blood mononuclear cells (PBMC) which could not be explained solely by the CCR5 genotypes of these individuals. Interestingly, the low levels of CCR5 on PBMC resulted in reduced levels of infection by M-tropic viruses, in vitro (42). Chemokine receptor expression is dependent, at least in part, on the activation state of the cell (44). Co-stimulation of T cells via the T cell receptor and CD28 resulted in down-regulation of CCR5 expression and induced a virus-resistant state (45).

Expression of CCR5 and CXCR4 on distinct subpopulations of PBMC, and their differential response to T cell activation may explain why M-tropic viruses are the species most often transmitted (43,44). In cases of sexual or parenteral transmission of HIV, M-tropic viruses are usually transmitted, even when the infecting population contained a mixture of M- and T-tropic viruses. Differences in the trafficking of naïve and memory T cell subsets may result in an increased probability that memory T cells, trafficking through skin, intestine and sites of inflammation, will come in contact with infiltrating virus. In addition, levels of CCR5 on memory T cell may be sufficient for infection, whereas levels of CXCR4 on these cells would not be sufficient for establishing an infection in the absence of activation.

In the brain, and CCR5 are expressed on microglia, and could be used as co-receptors by HIV for infection of CNS (46) resulting in the associated AIDS dementia. Eotaxin and a blocking antibody to CCR3 inhibited infection of microglia by HIV. Similarly, MIP-1β blocked infection of microglia via CCR5.

IDENTIFICATION OF NEW CHEMOKINE RECEPTORS AS HIV CO-RECEPTORS

Several novel HIV receptors have been identified recently. STRL33/TYMSTR is expressed on lymphoid cells and is induced in activated T cells (47,48). Cells that express both STRL33 and CD4 can be infected by both M- and T-tropic strains of HIV. The identity of the natural ligand for STRL33 is unknown as of this writing. US28, a β-chemokine receptor encoded by human cytomegalovirus, can serve as a co-receptor for HIV in $CD4^+$ cells (49), suggesting a means by which other viruses may play a role in HIV susceptibility. It was also found that SIV, the simian version of HIV, can use co-

receptors on PBMC other than CCR5 (50), suggesting that analogous receptors may be present in humans.

DEVELOPMENT OF CHEMOKINE RECEPTOR ANTAGONISTS AS THERAPY FOR TREATMENT OF AIDS

Chemokines and their receptors have, since their discovery, been appealing as drug targets (51). This area of research has only intensified since identification of CCR5 as the HIV co-receptor. Chemokine receptors are members of the 7-transmembrane, or serpentine, family of G-protein coupled receptors (GPCRs). By some accounts (52), small molecules targeting GPCRs represent some 50% of the approved drugs on the market. Although significant efforts are ongoing at many companies to develop small molecule antagonists of chemokine receptors, other entities including peptides, modified peptides, and antibodies could also be useful therapeutics. Notably, proof of concept demonstrating that a monoclonal antibody to CCR5 could block HIV infection, in vitro, has been achieved (42).

Little in the way of small molecule inhibitors of CCR5 has been reported to date. However, a derivative of the RANTES protein, aminooxypentane-RANTES or AOP-RANTES, containing a modification at the N-terminus, and possessing antagonist activity on CCR5 and CCR1, has been described (53). This modified chemokine blocked infection of susceptible cells by M-tropic HIV at subnanomolar concentrations. No agonist activity was noted. A similarly modified RANTES generated by recombinant techniques and containing an extra methionine residue at the N-terminus (Met-RANTES) showed similar antagonist activity (54).

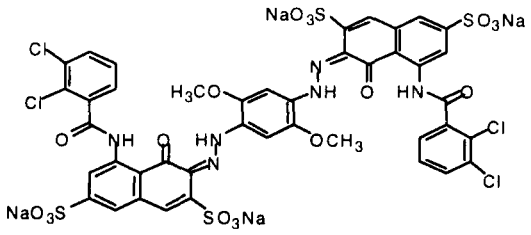
A synthetic peptide analog of polyphemusin II, called T22 ($Tyr^{5,12}$, Lys^{7})-polyphemusin II, blocked fusion and infectivity mediated by T-tropic HIV via the CXCR4 receptor (55). This 18 amino acid peptide analog also blocked binding of SDF-1 to CXCR4, and functional responses mediated by SDF-1 (Figure 2).

Recently, several groups have reported small molecule inhibitors of CXCR4. Using an in vitro fusion assay, a class of bis(disulfonaphthalene) dimethoxybenzene compounds (represented by FP-21399, Figure 2) has been identified that blocks HIV infection of $CD4^{+}$ cells (56). FP-21399 blocks both M- and T-tropic strains of HIV, and appears to be acting at the V3 loop of gp120. The compound is concentrated in lymph nodes, one of the major reservoirs of HIV during infection. In addition, the compound protected against HIV infection, in vivo, in SCID mice reconstituted with human immune cells.

Several labs have recently described results from AMD3100 (Figure 2), a member of the bicyclam class of compounds (57,58). This is a potent inhibitor of T-tropic HIV that appears to function via the CXCR4 receptor but no effects on infection of M-tropic viruses via CCR5 were observed. The compound blocked binding of SDF-1 and a CXCR4-specific monoclonal antibody to the CXCR4 receptor. AMD3100 also blocked functional responses including calcium flux and chemotaxis, in response to SDF-1, the natural ligand for CXCR4.

Another small molecule inhibitor, ALX40-4C (Figure 2) (N-a-acetyl-nona-D-arginine amide), also blocks infectivity of T-tropic and dual-tropic HIV-1 via the CXCR4 receptor (59). This compound, like the other CXCR4 antagonists, also blocked SDF-1 and monoclonal antibody binding to CXCR4. The report suggests that the compound blocks interaction of the V3 loop with the receptor. As this compound is a polyanion, it is possible that it interacts with the negatively-charged first and second extracellular

loops of CXCR4. This hypothesis is supported by the observation that this compound also blocks infection by HSV1, which is also sensitive to polyanions.



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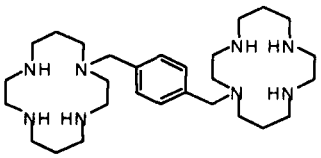
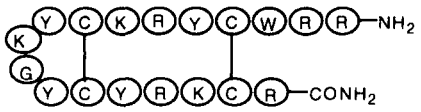
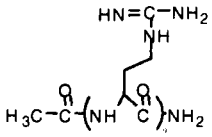


AMD3100



T22



ALX40-4C

Figure 2

Alternative therapeutic strategies include the search for agonists of CCR5 and CXCR4. Besides blocking virus binding, receptor agonists have the additional advantage in that they would also down-regulate the receptor. One potential flaw in this approach is that activation of the target cells could actually enhance the virus infection.

A therapeutic to block HIV interaction with CCR5 could have a major impact on viral transmission and viral load in infected individuals, but the potential impact of a CCR5 antagonist on viral evolution remains unclear. Based on the CCR5delta32 data, blocking CCR5 will attenuate HIV transmission. Additionally, it is assumed that a CCR5 antagonist will reduce the viral load in infected individuals, and is likely to have a beneficial effect initially. However, it is not clear this effect would be long-lasting, as it is possible that the selective pressure exerted by a CCR5 antagonist could lead to the outgrowth of SI forms of the virus, leading to more rapid progression to AIDS.

CONCLUSIONS

The identification of chemokine receptors as the long sought-after HIV co-receptor, that along with CD4, mediates the entry of viral particles into target cells, has opened up an entirely new approach for anti-HIV therapeutics and prophylactics. The initial findings have stimulated an extraordinary amount of work to elucidate the importance of CCR5 and CXCR4 as co-receptors as well as the mechanism by which co-receptor activity is achieved.

Structure function studies have demonstrated that the co-receptor activity is distinct from the more usual GPCR activity of the chemokine receptors in that signal transduction is not required for co-receptor activity. Rather, the chemokine receptor appears to be an anchor, along with CD4, that positions gp120 close to the membrane, and induces a conformational change in gp120 that exposes the gp41 fusion peptide. Additional studies indicate that the interaction of gp120 and CCR5 is complex and varies with HIV strains.

The CCR5delta32 polymorphism and the resistance to HIV infection of homozygous individuals provides strong evidence for a critical role of this receptor in HIV transmission and infection. That these individuals do not appear to suffer any deleterious effects due to the lack of functional CCR5 suggests that therapies designed to block CCR5 should not cause any immune system dysfunction. These findings have certainly lead to interest in the discovery of inhibitors of CCR5-gp120 binding for therapeutic use to reduce viral load and transmission.

Modified and native chemokines can serve as inhibitors of HIV utilization of CCR5, but these have limited clinical utility. Although, blocking monoclonal antibodies to both CCR5 and CXCR4 are available, molecule drugs with oral bioavailability would be much preferred. Several organic molecules that inhibit the co-receptor activity of CXCR4 have been described, but low molecular weight organic antagonists of CCR5 have not been reported.

All of the data available suggest that CCR5 is a very attractive target for therapeutics to block HIV transmission. However, it is still not clear whether blockade of CCR5 in newly exposed, or in infected individuals might lead to a more rapid evolution of M-tropic, NSI virus to T-tropic SI virus and contribute to a more rapid progression of disease severity. CCR5 blocking monoclonal antibodies, modified chemokine antagonists, and the early-stage CXCR4 small molecule inhibitors might provide reagents necessary to investigate this important question in in vivo model systems.

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Chapter 27. Roles of Caspases in Inflammation and Apoptosis: Prospects as Drug Discovery Targets

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Introduction - The caspase family of cysteine proteases has prominent roles in both cytokine maturation and apoptosis. Caspase-1 (interleukin-1β-converting enzyme; ICE), and probably other caspases, induce inflammation by proteolytic maturation of IL-1β and IL-18, and indirect activation of IL-1α and IFN-γ. The caspases are homologous to Ced-3, a *C. elegans* cysteine protease required for apoptosis, and many have signal transduction or effector functions in mammalian apoptosis. Although the roles and regulatory mechanisms of the caspases are incompletely understood, their activities suggest that specific inhibitors could be efficacious in several clinical indications.

THE CASPASE FAMILY: OVERVIEW

"Caspase" comes from cysteine protease with aspartic acid specificity (1). The family is defined by sequence homology and conservation of catalytic and substrate recognition residues. The human caspases are products of at least 10 genes (Table 1). Some may derive additional diversity from splice variation or isoforms. Their sequences suggest subdivision into three groups that share structural and functional properties. The primary function of the group A enzymes is cytokine maturation. Group B caspases 8, 9 and 10 are known to be the first caspases activated in apoptosis induced by different signals. These subsequently activate other caspases, and are considered apoptosis signaling molecules. Group C contains caspases that cleave a variety of substrates during apoptosis, and are considered downstream effectors.

SUBSTRATE SPECIFICITY

The substrate specificities of the caspases (Table 1) have been studied by combinatorial and defined-substrate methods (2,3). Like the apoptosis-inducing serine protease granzyme B (GrB), each enzyme displays a strong preference for Asp in P1. Also common is a preference for Glu in P3. The caspase-3/Ac-DEVD-cho cocrystal structure (4) shows that the Glu carboxylate contacts the guanidinium of a highly

Table 1. Caspase nomenclature and properties.

Caspase	Group	Trivial Names	Peptide Specificity	Probable Physiological Roles
1	A	ICE	W-E-x-D	Cytokine maturation, *e.g.*, IL-1β, IL-18
4		TX, Ich-2, $ICE_{rel}II$	(W/L)-E-x-D	
5		TY, $ICE_{rel}III$	W-E-x-D	
2	B	Ich-1	V-D-V-x-D	Apoptosis signaling, *e.g.*, caspase activation
8		MACH, FLICE, Mch5	L-E-x-D	
9		ICE-LAP6, Mch6	L-E-x-D	
10		Mch4, FLICE2	L-E-x-D	
3	C	CPP32, Apopain, Yama	D-E-x-D	Apoptosis effectors, *e.g.*, PARP or Lamin cleavage
6		Mch2	V-E-x-D	
7		Mch3, ICE-LAP3, CMH-1	D-E-x-D	

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conserved Arg, itself part of the P3 and P1 pockets. Analogous interactions may occur for all caspases. P2 specificity is broad, consistent with the inhibitor P2 side-chain solvent exposure observed for caspase-1 and -3 (4-6). P4 specificity differs substantially between the caspases, and can be categorized as aromatic, aliphatic or Asp. The degree of P4 specificity varies too; for example, caspase-2, -3 and -7 are highly specific for Asp, while caspase-8 and -9 accept residues from each category. In summary, P1 and P3 specificity distinguish caspases from other proteases, and P4 specificity differentiates members of the family.

The observed caspase specificities suggest the design of peptidic substrates and inhibitors. All except caspase-2 require that peptidic probes contain P1 - P4 residues (caspase-2 also requires P5) (3). While design of probes based on specificity has yielded efficiently recognized substrates and inhibitors, they are typically recognized by several of the caspases, presumably because of their overlapping specificities. Further efforts will be required to produce compounds sufficiently selective to dissect the individual roles of the caspases *in vivo*.

CASPASE REGULATION

Caspase regulatory mechanisms are of interest because they may offer opportunities for pharmacologic modulation of caspase activity. In addition, regulatory mechanisms may themselves be subject to dysfunction leading to disease.

Transcription and translation – The caspase-1 promoter contains a consensus interferon regulatory factor 1 (IRF-1) operator. IRF-1 is required for transcription of caspase-1 but not -2 (7). Another transcription factor, STAT-1 (signal transducer and activator of transcription-1), is required for constitutive expression of caspase-1 (8,9) as well as caspase-2 and -3 (8). However, pools of inactive caspase precursors are typically maintained in cells, and protein synthesis inhibitors are ineffective in blocking caspase activation. Thus gene activation is probably not rate-limiting in caspase activation, and so is not a promising drug discovery target.

Posttranslational regulation – The rate-limiting step in caspase activation appears to be proteolytic activation. As described below, proteolytic caspase activation can be stimulated by induced proximity of caspase precursors or proteolysis by active caspases or other proteases. In activated human monocytic THP.1 cells, active caspase-1 has a very short half-life, being present at <1% of the level of precursor enzyme (10). *In vitro*, caspase-1 autodegradation at Asp381 (11) and reversible subunit dissociation (11,12) readily occur. In addition to being a potential contributor to inactivation, dissociation may allow rearrangement between different caspases. Murine caspase-11, probably equivalent to human caspase-4 or -5, physically interacts with and is required for murine caspase-1 activation (13). The *in vitro* observations of caspase precursor interdigitation (14) and of a reversible homodimeric equilibrium (11,12) suggests that formation of heteroligomeric species containing portions of up to four caspases is possible (11).

Nitric oxide – NO is a thiol-modifying agent produced by three known nitric oxide synthases (NOS). NO inhibits all caspases *in vitro* (15) in a redox-reversible manner. Caspase activity in apoptotic mammalian cells is blocked by NO donors (16-18). The liver-specific NO donor V-PYRRO/NO blocked TNF-α/galactosamine-induced apoptosis and hepatotoxicity in rats (19), setting a precedent for pharmacologic modulation of caspase-induced apoptosis in this manner. The antiapoptotic effects of shear stress (20) or a cytokine mixture (21) on cultured mammalian cells is blocked by NOS inhibitors. Fas-apoptosis is potentiated by NOS inhibitors (22). Thus, constitutively produced NO can negatively regulate apoptosis *via* caspase inhibition.

Apoptosis-inhibitory proteins – Several antiapoptotic viral proteins have been described. Baculovirus p35 (23) and cowpox virus crmA (24) are serpin-like proteins

that inhibit caspases by direct binding (24,25). No human homologs of these are known. Adenovirus encodes a 14.7 kDa protein that interacts with caspase-8 and prevents Fas-induced apoptosis (26). Another group, the IAPs (inhibitor of apoptosis) (27), is unrelated to serpins in sequence. Several human IAPs have been reported. Of these, XIAP (28) and c-IAP-1 and -2 (29), inhibit caspases directly. Each displays substantial selectivity, potently inhibiting caspase-3 and -7 but not -1, -6 or -8. The functions and relationships of the human IAPs, and the significance of the selectivity of the three that inhibit caspases, are of continuing interest. A caspase-8 and -10 homolog, first called I-FLICE (30) and subsequently described by at least five other groups, lacks the caspase catalytic residues. This protein may inhibit by rearrangement with active caspases as described above.

CASPASE FUNCTION IN CYTOKINE MATURATION

Proinflammatory cytokines – Caspase-1 cleaves the prodomain from inactive pro-IL-1β at YVHD(119)-A, producing bioactive IL-1β (31,32). Characterization of caspase-1-deficient mice confirmed that caspase-1 plays a major role *in vivo* in activation of IL-1β (33,34). Unexpectedly, these mice also had a partial defect in IL-1α production, which is not fully understood. Caspase-1 does not cleave pro-IL-1α (35); instead, a calpain family protease may process IL-1α (36,37). We believe that caspase-1 cleaves the endogenous calpain inhibitor calpastatin and thus stimulates calpain-mediated processing and release of mature IL-1α. Caspase-1-deficient mice also are defective in LPS-induced interferon-γ (IFN-γ) production (38,39). This results from an essential role of caspase-1 in IFN-γ-inducing factor (IGIF or IL-18) maturation. IL-18, among other activities, stimulates IFN-γ production from NK and T cells, and synergizes with IL-12 in inducing IFN-γ production (40). Caspase-1 cleaves murine pro-IL-18 at LESD(35)-N to generate bioactive IL-18. Caspase-4 can also process pro-IL-18 *in vitro*, and may do so *in vivo*.

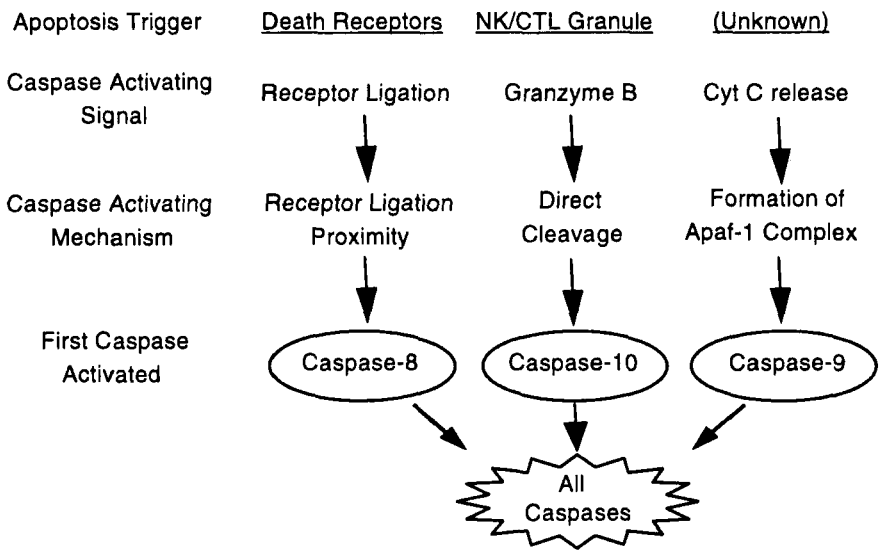
Other cytokines – It had appeared likely that caspase involvement in cytokine maturation is restricted to members of the group A subfamily. However, a recent study (41) indicates that pro-IL-16 is a substrate of caspase-3 (but not caspase-1 or GrB), which cleaves at the site SSTD(253)-S to generate bioactive IL-16. Further work is needed to determine whether caspase-3 or others process IL-16 *in vivo*.

CASPASE ACTIVATION IN APOPTOSIS

Diverse apoptogenic stimuli cause a similar activation of the caspases, a common feature of apoptosis. While the signaling events for most such stimuli are not understood, three have now been described in at least partial detail: Fas/FasL activation of caspase-8 and possibly caspase-10, GrB activation of caspase-10, and cytochrome c/Apaf-1 initiated activation of caspase-9 (Figure 1).

Fas activation of caspase-8 – Fas (CD95/APO-1) is the best understood member of a family of "death receptors" (DR) including TNFR1, DR3, DR4 and DR5 (42). Inactive pro-caspase-8 (and probably pro-caspase-10) interacts with the adapter FADD by homologous "death effector domains" (43) on each protein. Fas ligand (or agonistic antibody) binding induces receptor trimerization. This recruits the FADD/caspase-8 complex, interacting by FADD and intracellular Fas "death domains". The resulting proximity of two or more pro-caspase-8 molecules promotes enzyme cross-activation and release (43,44). Similarly, caspase-2 also associates with Fas, *via* the adaptor RAIDD/CRADD (45,46), and may also be activated directly by DRs. In parallel to direct caspase-8 activation, Fas apoptosis can also proceed by activation of Jun N-terminal kinase (JNK) *via* the adaptor Daxx (47), followed by caspase activation.

Figure 1: Three Mechanisms of Caspase Activation in Apoptosis



Granzyme B activation of caspase-10 – In addition to Fas, the major mechanism by which natural killer (NK) and cytotoxic T lymphocyte (CTL) cells kill tumor, virus-infected and non-self cells is granule-mediated delivery of perforin and several serine proteases, principally GrB. While GrB can kill target cells independently of caspases (48), its principle mechanism appears to be proteolytic caspase activation. GrB can activate several caspases, but kinetic studies show that caspase-7 and -10 are strongly preferred substrates (48). In cell culture models, full caspase-7 maturation (*i.e.*, cleavage at both of its proteolytic maturation sites) requires other active caspases (48), suggesting that caspase-10 is the primary target for GrB-initiated apoptosis.

Cytochrome c initiated activation of caspase-9 – A third caspase activation mechanism during apoptosis is controlled by mitochondrial release of cytochrome c (cyt c). It is not known which of many proposed apoptosis signals lead to cyt c release in particular (49,50). Downstream events, however, have been elucidated. Three apoptotic protease activating factors (Apaf-1, -2 and -3) were required to reconstitute caspase-3 activation in a cell-free system (51). Apaf-2 was identified as cyt c (51), and Apaf-1 (52) as a mammalian homolog of Ced-4, a *C. elegans* gene product essential for cell death (53,54). Apaf-3 was later identified (55) as caspase-9. Cyt c binding to Apaf-1 in the presence of ATP/dATP induces binding and activation of caspase-9, which itself then proteolytically activates caspase-3 (55).

Downstream caspase activation – In each case, an apoptogenic stimulus preferentially activates one or two distinct caspases, followed by general activation of other caspases. Caspase specificity matches caspase maturation site sequences well, and auto- and cross-activation is promiscuous. All caspases can induce apoptosis when overexpressed in mammalian cells, arguing against a requisite initiating role for any particular caspase. Thus, after crossing a threshold controlled by one or more initiator caspases, activation of the other caspases in the final phase of cell death probably occurs in an explosive, multiply parallel manner rather than as a stepwise cascade.

CASPASE FUNCTION IN APOPTOSIS

Identification of the essential features of apoptosis is a matter of considerable current effort. Apoptosis is an orderly dismantling of unwanted cells, leaving no end product. As such, adventitious events that do not interfere with the process might not

be selected against, and might be misleading. New putative caspase substrates are cleaved with a wide range of catalytic efficiencies, and skepticism is warranted in judging which are requisite steps in apoptosis and which are *in vitro* artefacts. Many can be absent in cells that nevertheless display normal apoptosis (56). Still, the consequences of cleavage of many identified caspase substrates logically contribute to common events in apoptosis. These can be categorized as DNA metabolizing (repair), structural, and signal transducing molecules. Following and in Table 2 is a summary of some of the known caspase targets, and how their cleavage might contribute to apoptosis.

DNA metabolizing enzymes – A hallmark of apoptosis is DNA degradation, and a proposed caspase role is inactivation of the DNA repair proteins that would otherwise oppose it. One of the first identified and most commonly observed caspase substrates is poly(ADP-ribose) polymerase (PARP). PARP recognizes DNA single strand breaks, and by poorly understood mechanisms, initiates repair by other factors (57). One of the PARP cleavage fragments may recognize and interfere with DNA double strand break repair (58-60). The catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}), which recognizes DNA double strand breaks and signals for their repair, is also a caspase substrate (61,62). Cleavage inactivates the kinase, but like PARP, the DNA binding subunit (Ku) is not destroyed and may interfere with DNA repair.

An important recent discovery was the murine caspase-activated deoxyribonuclease (CAD), and its associated inhibitor ICAD (63) and their human orthologs, DNA fragmentation factor-40 (DFF-40, corresponding to CAD) and DFF-45 (corresponding to ICAD) (64). CAD catalyzes the DNA fragmentation commonly observed in apoptosing cells. Caspases activate CAD by proteolytically inactivating ICAD (65).

Structural proteins – The common morphological features of apoptotic cells can be attributed to changes in structural proteins. Lamins, which maintain structural integrity of the nuclear envelope, are cleaved in many apoptotic cells. Caspase-6 is probably the primary lamin-cleaving caspase (66,67), since its substate specificity matches the site cleaved during apoptosis (2,3,66,67). Both inhibitor studies in apoptosing cells (68-70) and *in vitro* cleavage assays (71) suggest that fodrin is cleaved by caspases. Caspases also have a role in regulating apoptotic plasma membrane changes resulting in phagocytic removal of apoptotic cells (72,73).

Signalling molecules – Caspase-initiated signal transduction pathways and the actions that they trigger remain poorly understood. Some caspase substrates hint at the nature of those pathways. An intriguing example is SREBP, a membrane-bound sterol-binding transcription factor that is released from the membrane by caspase cleavage and translocates to the nucleus (77). Protein kinase Cδ is an enzyme that is activated by caspase cleavage (79,80), in contrast to many that are inactivated. The cell cycle regulatory retinoblastoma (Rb) protein, in its hypophosphorylated form (84,85), and the antiapoptotic protein Bcl-2 (88), are also cleaved by caspases during apoptosis. In

Table 2. Examples of Caspase Substrates in Apoptosis

DNA Metabolism		Structural Proteins		Signalling Molecules			
Target	Ref.	Target	Ref.	Target	Ref.	Target	Ref.
PARP	(74,75)	Lamins	(58,76)	SREBP	(77)	PITSLRE	(78)
DNA-PK	(61,62)	Fodrin	(68-71)	PKC-δ	(79,80)	D4-GDI	(81)
DFF-40, 45	(63-65)	Actin	(82,83)	Rb	(84,85)	PRK2	(86)
		Gas2	(87)	Bcl-2	(88)	MEKK-1	(89)
		Gelsolin	(90)	U1-70 kDa	(91)	Ras-GAP	(92)

both cases cleavage should favor apoptosis.

CASPASE INVOLVEMENT IN DISEASE

The biological roles of caspases in both cytokine production and apoptosis are relevant to human disease. In terms of caspase regulated cytokines, IL-1 involvement in disease has been reviewed extensively (93,94). Caspases as therapeutic targets for diseases that involve inappropriate apoptosis has also been reviewed (95). Here, we discuss more recent data, in particular from experiments using gene knockout mice and caspase inhibitors in animal models of injury and disease.

Inflammation - Caspase-1-deficient mice have provided strong evidence for caspase-1 involvement in two models of acute inflammation and injury. The mice are resistant to endotoxic shock in a high dose LPS-induced lethality model (33). Interestingly, IL-1β-deficient mice are susceptible to high dose LPS shock (96,97). Septic shock pathogenesis is not well understood even in animal models, but caspase-1-deficient mice probably survive because of reduced production of the proinflammatory cytokines IL-1α, IL-1β, IL-18 and IFN-γ. Clearly, it will be of interest to determine whether caspase-1 inhibitors are protective in sepsis models in other animals. More recently caspase-11-deficient mice were characterized and are resistant to high dose LPS shock (13). The human equivalent of murine caspase-11 has not been identified; human caspase-4 and -5 are most homologous. Caspase-11 associates with and has an essential role in murine caspase-1 activation.

Stroke - The second acute injury model in which caspase-1-deficient mice were tested was the middle cerebral artery occlusion (MCAO) model for stroke (98). Brain edema and the extent of brain damage resulting from MCAO were significantly less in caspase-1-deficient mice than that of control C57BL/6 mice, despite similar reductions in blood flow. Similar results were obtained in the MCAO stroke model using transgenic mice expressing a dominant negative caspase-1 mutant (99). In addition, irreversible peptidic caspase inhibitors reduced brain damage in the MCAO stroke model in mice and rats (100,101). The inhibitors used in mice were *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-fmk) and *N*-benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (z-DEVD-fmk), administered by intracerebroventricular injection (100). The inhibitors displayed differences in biological activity, in particular z-DEVD-fmk did not reduce ischemia-induced IL-1β levels in the brain whereas z-VAD-fmk did so significantly. These observations are relevant to the mechanism(s) by which the caspase inhibitors are protective in the MCAO model. A open question is the relative contributions of blocking IL-1 and IL-18 production by caspase-1 inhibition, *vs.* inhibition of other caspases that regulate neuronal apoptosis. This is a controversial area both in terms of possible involvement of caspase-1 in apoptosis and relevance of apoptosis in ischemic brain damage. More selective caspase inhibitors will be required to investigate this issue further.

The observation that in mice, a dominant negative inhibitor of caspase-1 slowed the progression of amyotrophic lateral sclerosis (102) suggests that caspase-1 inhibitors might be effective treatments for this and other neurodegenerative diseases. As noted above, the relative contributions of caspase-1 in cytokine maturation *vs.* activation of apoptosis remains unclear.

There is considerable interest in therapeutic targeting of caspases, other than caspase-1, to inhibit apoptosis in indications including stroke, myocardial ischemia and neurodegenerative disease. z-VAD-fmk protects mice against Fas-mediated liver damage and death (103). The liver damage is due to massive apoptosis of hepatocytes induced by Fas receptor activation. Protection by z-VAD-fmk in this model system is encouraging, but it should be noted that the hepatocytes are normal healthy cells prior to induction of apoptosis. Where cells have already sustained damage,

inhibition of apoptosis may not prevent cell death or be beneficial. These questions can be addressed by further caspase inhibitor testing in animal models of acute injury and disease. However, the use of caspase inhibitors in chronic settings, such as neurodegenerative diseases, raises additional concerns about potential inhibition of normal apoptotic events. Caspase-3-deficient mice (104) die at 1 to 3 weeks of age. They appear to succumb to defects in apoptosis that are restricted to the brain. Thymocytes from mutant and wild-type mice were equally sensitive to induction of apoptosis by several stimuli. These results could be interpreted to favor the idea that therapeutic blockade of neuronal apoptosis may be achieved without inhibiting apoptosis in the immune system. It will be important to see whether other caspase family members function in apoptosis in a tissue or cell-type restricted manner. If other caspases have essential roles in apoptosis during development, then conditional gene knockouts may be required for testing function in animal disease models.

SUMMARY

Caspase-1 is a validated therapeutic target for the treatment of inflammation. In chronic inflammatory diseases, selectivity of inhibitors for caspase-1 is important. For example, caspase-8 cross-inhibition would be unacceptable because of likely autoimmune consequences. For acute inflammation and injury, as in sepsis and stroke, a less selective caspase inhibitor capable of blocking both caspase-mediated cytokine production and apoptosis might be desirable. Possible caspase targets for inhibition of apoptosis in acute settings, such as ischemic injury, include caspases-3, -7 and -9. In addition, because of their roles in Fas- and GrB-mediated apoptosis, graft rejection due to NK- or CTL-mediated cell killing might be retarded by inhibition of caspase-8 and -10. We note, however, that our understanding of the roles of the caspases in apoptosis is increasing rapidly, and the list of the most promising therapeutic targets will most likely change with it. In neurodegenerative diseases, selection of caspase targets is even more speculative. These uncertainties do not change the conclusion that caspase inhibition may be effective in many clinical indications for which current therapies, if any, are limited.

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SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

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Chapter 28. Predictive Toxicology: An Overview

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Introduction - Viewed simplistically, there are three major aspects to the field of *in vitro* toxicology today: 1) models already available to address specific issues, 2) validating and bringing into more extensive use those assays with potential to reduce reliance on an *in vivo* test, particularly in a regulatory setting, and 3) utilizing cutting-edge tools to refine and extend in vitro models. This chapter will attempt to give the reader examples of current models and an overview of validation for replacement of animal tests (an area where scientific and regulatory needs are being merged) as representative of where the field is today. But it will concentrate on the third aspect, where the science is focused on the refinement of models and the exploration (and application) of newer tools, because it is this arena that will produce the next generation of models and shape the future of predictive toxicology.

TARGETS AND ENDPOINTS

For many scientists the task at hand involves understanding a relatively well defined question. What is the mechanism by which a particular class of compounds exerts its toxicity? Can one predict the relative toxicity or pharmacologic response of a specific group of materials? Does compound X induce the same profile of metabolic enzymes across species? To address these types of questions, specific methods are necessary, and this is an area where an understanding of tissue culture models has been coupled with various endpoints, particularly those related to cellular cytotoxicity. The literature is replete with protocols using a multitude of target tissues and endpoints, some of which will be described below (cf., 1).

Targets - The choice of target tissue for predictive toxicology runs the gamut from isolated perfused organs through tissue slices to single cells, both freshly isolated and maintained as lines. Some of the key issues in using various tissues for evaluating target organ toxicity have been summarized using the kidney as an example (2). For example, irrespective of which target one chooses, it is critical to evaluate the biochemistry of the tissue; are the expected tissue-specific enzyme activities or other biochemical markers maintained? The authors demonstrated this point by comparing transport, ATP levels, marker and metabolic enzymes among cell lines and primary cells from a number of species. After establishing the best model of the *in vivo* situation for their purposes, they went on to assess the effects of gentamicin and platinum compound exposure. They evaluated the viability and maintenance of markers in the various models and found that these are maintained longer in single cell cultures. Thus when the goal is to mimic chronic exposures, cultured cells are more suitable. For mimicking acute exposures, perfused organs, slices, or tissue fragments may provide a better reflection of the *in vivo* situation.

Although isolated, perfused organs are relatively short-lived and, it is difficult to perform a number of replicates simultaneously (because of the technical complexity of isolation), one can address some specific mechanistic questions more efficiently with

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this system relative to cell culture models. For instance, evaluation of the vasoconstrictive effect of cyclosporine A and its reversal by L-propionylcarnitine, in perfused rat kidney, would probably not be possible in any other *in vitro* system (3). The histological assessment of kidney damage was shown to correlate well with the observed vasoconstriction in this study, further confirming the validity of the model. Cyclosporine-induced effects on bile flow in perfused rat liver have also been modeled (4). This study also followed release of cytosolic and mitochondrial enzymes into the perfusate as a marker of toxicity (mimicking clinical monitoring of enzymes in the plasma).

Tissue slices, particularly precision-cut slices, represent a way of maintaining the three dimensional cell-cell associations in a system which is more accessible in terms of markers of toxicity (which do not have as far to diffuse compared to a whole organ system) or mechanisms of action (where the specific cell types involved in a response may be identified). On the downside, slices cannot easily be maintained in culture for long periods of time, and some of the tissue is damaged by the cut, so care needs to be taken that this does not interfere with the measured endpoint. Only a few examples will be presented here, since a good overview of this model has been published (5). One example (of many) of validation of liver slices for the study of metabolic enzymes is work showing that the induction of CYP1A1 and CYP1A2 by TCDD in slices and in *in vivo* rat liver correlate very well (6). This study points up another advantage of tissue slices which is the ability to use human material. A case in point is a study examining the metabolism and toxicity of cyclosporine A in human liver and kidney slices (7). In this work, the author was able to identify potential clinical markers for cyclosporine-induced effects from observations made with the slices. The distribution of 11 beta-hydroxy steroid-oxido reductase in multiple organs (liver, testis, rectum, kidney, lung) from rat, compared with the enzyme activity seen in placental homogenates and microsomes from different tissues in different species further highlights the versatility of the technique (8).

'Reconstructed' tissues (or constructs) represent a model intermediate between slices or perfused organs and cultured cells. In general the cell type(s) considered critical for the model are allowed to differentiate and re-form cell-cell interactions in an appropriate *in vitro* environment. These constructs can generally be maintained longer than slices, and may arguably be more stable and reproducible for longer term studies. A number of skin models exist and have been used for toxicity and metabolism (9, 10). A model of cornea has also been reported for prediction of eye irritation (11).

Both cell lines and primary isolates of cells have been used for toxicity and biochemical studies for quite some time. Cell lines, frozen down at a specific passage, enable one to conduct multiple experiments over time on essentially the same cell population. Clearly, though, cells adapted to culture and carried for long periods of time are not identical to primary cells and, while retaining some characteristics of the organ of origin, by no means do they retain all these characteristics. Thus care needs to be taken in equating the results to the *in vivo* or primary cell situation. Hepatocytes are probably the most widely used primary cells and can be maintained in culture with functional CYP enzymes, though for a relatively brief time. Metabolism studies therefore are limited to acute exposures. However, the use of cells genetically engineered to express individual CYP enzymes is extending the ability to evaluate metabolism questions with cells in culture. An example is the development of lymphoblastoid cells (12) and human colon tumor-derived Caco-2 cells (13) which stably express human CYP enzymes and which may, therefore, more accurately reflect human metabolism. And one application of

genetically engineered cells is the demonstration that CYP1A1 plays a major role in the metabolism and enhanced genotoxicity of the flavonoid kaempferol using V79 (Chinese hamster lung) cells engineered so that each line contained a single rat CYP (14).

Endpoints - The most common measurement(s) made in *in vitro* tests are of cytotoxicity. The diversity of cytotoxicity tests is illustrated in a survey of tests applied to cutaneous and ocular irritation, which shows that measurements have been made of such diverse endpoints as cell number (growth), macromolecular content, enzyme leakage, and membrane integrity, among other parameters (15). All are well recognized measures of xenobiotic-induced cell death, which tend to measure population effects, i.e., generalized killing of cells by some fundamental mechanism common to all cells. This type of test has been used frequently as a first level screen, to choose among analogs or formulations, or to predict the potential for irritation or toxicity. A good reference book on general applications of cytotoxicity endpoints has been published (1).

When the goal is to gain an understanding of mechanism(s) of toxicity or action, earlier, more subtle, or more specific endpoints, (e.g., cell type specific or functional) need to be used. For instance, a study examining kidney toxicity, in addition to evaluating generalized toxicity by total protein measurement, also took into account Na+/K+-ATPase and α-methyl glucopyranoside uptake as indicators of transport characteristic of proximal tubule cells (2). The development of various fluorimetric probes specific for different biochemical processes has also proven useful to gain more mechanistic understanding. A good review on fluorescent probes, covering the rationale, methods, and caveats for indicators of membrane integrity, intracellular Ca^{++} and pH, mixed function oxidases, etc. has been published (16).

ASSAYS IN USE

Targeted Assays - The current state of the art of some *in vitro* assays, though, allows us to consider whether we might use them more generally as replacements for or complements to specific animal tests in an interlaboratory or regulatory situation. Evaluation, validation, and acceptance are major hurdles in this process, and part of the function of groups such as ECVAM (the European Centre for the Validation of Alternative Methods), ICCVAM (Interagency Coordinating Committee for Validation of Alternative Methods), and CAAT (Center for Alternatives to Animal Testing) is to assist in this process.

In seeking to move the field of *in vitro* toxicology forward, ECVAM has held a number of focused workshops which have critically examined alternative methods from both a technical and practical point of view. These reports provide overviews of the state of the art of various *in vitro* assays in such areas as quantitative structure activity relationships (QSAR) and physiologically based pharmacokinetic (PBPK) models (17, 18) and methods for assessing toxicity in target organs such as kidney (19), lung (20), skin (21, 22), and nervous tissue (23), and will serve as a good general starting point for the reader interested in applying these methods.

A specific area where much work has been done addressing animal replacement for regulatory purposes is in evaluating *in vitro* and *ex vivo* methods as potential replacements for the Draize eye irritation test. These are examples of tests developed with a specific goal in mind, and some of these assays have been found effective enough to be used for in-house decision making with defined sets of compounds, even though they may not be accepted for screening of broad chemical classes or as a

regulatory substitute for animal tests as yet. Evaluation of the many tests which have been proposed as Draize alternatives has narrowed the field and the most promising assays are being subjected to more rigorous inter-laboratory and regulatory validation efforts (24-27).

Validation - Validation is an area which has been extensively discussed in the literature, but will not be the subject of this chapter for the simple reason that when speaking of predictive toxicology, many researchers are not dealing with global validation, but rather in-house evaluation, asking the question of whether a model system suits their needs for the specific, usually narrowly defined question at hand. While some of the same points apply (for instance, does the test correctly rank chemicals on the basis of cytotoxicity or efficacy, how reliable and reproducible is it, how can the resulting data best be used in choosing among analogs or formulations in development, or in predicting human risk), an in-house evaluation is usually less extensive. The reader who is interested in points to consider with respect to validation and regulatory acceptance should consult one of the many manuscripts published on this subject (cf., 28-30).

MOLECULAR TOXICOLOGY

The third aspect to predictive toxicology is the continuing refinement of current *in vitro* models and the development of additional endpoints. It should be obvious that in designing model systems and endpoints for screening, one can, and must, address specific questions such as mechanism(s) of toxicity, metabolism, and/or drug distribution. And as our knowledge in these areas grows, both models and endpoints become more sophisticated and new applications are defined. The development of new technology coming mainly from the field of molecular biology is currently allowing greater understanding and new applications of existing models. This represents the marriage of the relatively well developed area of tissue and organ culture with the newest techniques of molecular biology. Endpoint measurements of general viability or function of cell populations are being supplemented by the growing ability to evaluate changes in gene expression which underlie cell death or dysfunction. A brief overview of some of these tools and their application to toxicology has been the subject of a recent review (31).

RT-PCR - Although one can look at DNA, RNA or protein to evaluate what is taking place inside a cell, it is usually more straightforward to look at mRNA expression as a surrogate for DNA or protein levels (although as discussed below this may be misleading in some cases). However, to visualize the mRNA and compare levels between, for example, control and treated, the signal needs to be amplified. Perhaps the most commonly used technique to accomplish this is the reverse transcription-polymerase chain reaction (RT-PCR). Detailed procedures for RT-PCR, as well as applications, have been published (cf., 32). In brief, total RNA is extracted from the cells under investigation, and a reverse transcription reaction is performed to produce cDNA. This cDNA can then be amplified in a PCR reaction until it can be visualized on an acrylamide gel. The resultant bands are compared (treated and control, different times of exposure, etc.) to establish relative changes in intensity. Since the amount of cDNA visualized is proportional to the amount of mRNA in the starting sample, an increase in the density of a DNA band suggests that the particular gene was induced.

Quantitative PCR - RT-PCR is considered a semi-quantitative technique. Although useful for demonstrating large changes in gene expression, the method is more problematical when changes of 2-4 fold are involved. In searching for ways to make

PCR more quantitative, methods have been developed which include use of an internal standard (32). For instance altered CYP1A1 induction by TCDD in rats was detected at an earlier time by quantitative RT-PCR than by measurement of ethoxyresorufin-o-deethylase activity (33). Additionally, the sensitivity of the technique was demonstrated by the ability to detect just a fivefold increase in UDP-glucuronosyltransferase above a substantial constitutive level. An internal control has been used in both the RT and PCR steps in the quantitative measurement of constitutive as well as Con A-induced cytokines in Th1 and Th2 cell clones, demonstrating the high degree of sensitivity and accuracy compared with routine RT-PCR (34).

Another method currently being developed is based on the inclusion of fluorigenic probes in a standard PCR reaction (35). This method promises detection capability for relatively small changes in gene expression, which, although minimal, may still have biological significance. A probe, with a fluorescent dye at one end and a quencher at the other, hybridizes to a site on the target gene between the two primers being used for PCR. As Taq polymerase extends the target gene in the PCR reaction, its 5'-3' nuclease activity cleaves the molecule of dye on a hybridized probe, releasing it from proximity to the quencher and thus allowing its fluorescence to be detected. In theory, each copy of the target gene made results in the release of one fluorescent unit. By determining the number of the PCR cycle at which fluorescence signal is first detectable in an unknown sample and comparing it with standards of known copy number, one has a quantitative measure of how much of the target was in the starting sample.

An application of this methodology demonstrated that the procedure can detect hepatitis C virus RNA in chimpanzee plasma samples with equivalent sensitivity, while being faster, more reliable, and giving higher overall throughput (36).

In situ PCR/Molecular Beacons - Although PCR is quite powerful in detecting altered genes in a population of cells, in an organ composed of heterogeneous cell types, it is difficult to know which cell type(s) is responding. *In situ* PCR, a developing technique which is as yet reportedly difficult to optimize, may yield an approach to this problem. Frozen or paraffin embedded tissue, tissue slices, or cells are attached to glass slides then subjected to PCR in a specially designed thermocycler. When the primers are labeled, for instance with fluorescein, the PCR amplified product is detectable at its *in situ* location by microscopy. An example of the application of this technique is its use to confirm the integration of the TNF gene into a mammary carcinoma cell line by a retroviral vector (37). Non-transduced cells treated with DNase to remove the endogenous TNF gene showed no signal after *in situ* RT-PCR. Transduced cells, on the other hand, showed a bright fluorescence after the same treatment indicating that mRNA had been expressed in the cells and could be amplified by the *in situ* RT-PCR method.

Another developing technique for visualizing differential expression of genes *in situ* is the use of 'molecular beacons' (38). Briefly, a probe that will hybridize to the gene of interest, is made with a fluorescent tag at one end and a quencher at the other. In this case, however, the reporter molecule and quencher are held in close proximity by virtue of the bases just inside these two molecules being complementary and forming a hairpin structure. When the probe is heated to hybridization temperature in the presence of the target tissue to be probed, the hairpin structure comes apart. If the probe hybridizes to a target DNA, quenching is lost and the molecule fluoresces, 'tagging' the cell in which the gene of interest is being expressed. Unhybridized probes reform the hairpin structure and are quenched as the

temperature is lowered, and thus only hybridized probes generate signal. While sensitivity may be an issue for *in situ* localization, the potential power of the method should be obvious.

Differential Display of mRNA - The RT-PCR techniques discussed above are quite useful for detecting specific genes, and indeed require knowledge of specific gene sequences for primer selection. But if the question is broader, if one asks which genes (known or unknown) might show increased or decreased expression under various conditions, another approach needs to be taken. Differential display of mRNA is one technique that addresses this issue (39). Briefly the method entails using defined pairs of primers, 'anchored' to the 3' poly-A sequence downstream and arbitrary on the upstream end, in each PCR reaction. The fragments generated from the populations to be compared are run side by side on polyacrylamide gels, and the gels are examined for changes in the banding pattern produced. The altered bands, representing genes differentially regulated, can be cut out of the gel, sequenced, and compared with sequences in databases such as GenBank for identification. As originally reported, 240 separate primer sets would need to be analyzed to theoretically visualize fragments of every mRNA in the cell population at a given point (during differentiation, after exposure to xenobiotic, etc.), a not insignificant effort. However, as interest in the technique grows, modifications of the methods are making differential display less labor intensive (40). One example of application of differential display is a comparison of different gene expression patterns among normal melanocytes, a primary melanoma cell line, and a metastatic melanoma cell line. (41). Of 42 genes identified as differentially expressed, many were associated with ribosomal and mitochondrial proteins which were upregulated in the tumor cells, or genes associated with regulation of the immune response. Fifteen fragments showed no significant homology with known genes. Thus, without a priori knowledge of the genes to be examined, these investigators have identified genes to target in more definitive studies.

Gene/protein Expression Matrices - Another approach to determining which endpoints to evaluate without specific a priori knowledge is by evaluating panels of genes for expression. The expression of groups of related gene families are likely to be altered by, for instance, exposure to xenobiotics, differentiation status, or disease states. Therefore screening a panel of genes increases the chance that products critical to the process under investigation will be identified. Illustrative of this approach is the screening of stress genes (summarized in 42). In this method, an identification was made of genes that were believed to cover the major pathways involved in response to stress. These genes were then put into constructs with a promoter and reporter so that induction could be easily quantitated. The results of this broad screening, particularly when compared with the profile induced by other materials, is presumed to be indicative of the mechanism by which the stressor is acting. Along the same lines, a battery of inflammatory cytokines elicited by contact irritants, sensitizers, and ulcerative agents applied to mouse skin was identified (43). It was hoped that examining cytokine induction profiles would lead to the identification of critical molecules specific to each type of insult.

Another developing method for broad examination of the effects of xenobiotics lies in the emerging technology of DNA microarrays. Briefly, arrays of 20 basepair oligonucleotide fragments of known genes are attached to a solid support (the chip) with densities ranging from approximately 100 to over 1000 genes/chip. RNA isolated from either *in vitro* or *in vivo* sources is amplified and tagged with an indicator, such as biotin. When the RNA is incubated with the chip, the extent of hybridization is indicative of the expression of specific genes. The power of this

technique lies in the ability to screen for hundreds of genes (limited only by their representation on the chip) in an individual sample. For instance, high-density arrays have been used to evaluate polymorphisms in the human mitochondrial genome (44). Mutations associated with a particular disease (Leber's hereditary optic neuropathy) were identified, and polymorphisms were compared among Caucasian and non-Caucasian individuals. Another application to the study of diseases is seen in the use of both low and high density arrays to assess the involvement of various genes in rheumatoid arthritis (45). The lower density arrays were composed of genes already suspected to be involved in the disease, providing a confirmatory and screening tool, while the higher density arrays represented over 1000 genes from a peripheral blood lymphocyte library, providing an exploratory tool to identify molecules not previously associated with the disease.

Since it is proteins which actually put the DNA instructions into effect, some groups feel that evaluation of protein changes is critical for assessing the cellular response to stress. In fact, some researchers have estimated that due to post-transcriptional regulation, as many as half of induced mRNAs in a cell may not correlate with increased levels of the proteins they encode (46). Attempts have been made to evaluate protein profiles during cellular response to xenobiotics, though high throughput methods to identify proteins represent a technical challenge (47). For instance, in an evaluation of the protein changes seen in mouse liver after exposure to peroxisome proliferators, it was found that while a number of protein changes were identified as relevant to the peroxisome proliferator effect, many of these protein spots could not be identified, making interpretation of the results problematic (48).

SUMMARY

It should be obvious from the above discussion that the field of *in vitro*, predictive toxicology is quite diverse. In-house use of non-whole animal methods is growing as the pressure increases for higher throughput screens in product development and for increased understanding of specific mechanistic questions. And utilization of these models will likely increase as developments in both *in vitro* maintenance of target tissue and tools based on molecular biology enhance our ability to address these issues at a more sophisticated level. In addition to being used in academic and industrial laboratories, *in vitro* methods are moving into the regulatory arena, where some assays are undergoing rigorous validation as potential replacements for specific animal tests. As newer techniques and increasing amounts of data are generated by efforts such as the Human Genome Project, our ability to predict toxicity, metabolism, etc. will depend on the continued refinement of *in vitro* models.

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Chapter 29. The Use of Surface Plasmon Resonance Based Biosensors In Drug Discovery

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Introduction - The modern drug discovery platform is defined by the characterization of interactions between biologically relevant molecules. These molecules can be proteins, oligonucleotides, carbohydrates, or even small molecules like hormones. In today's world of biotechnology, the therapeutic agent could be any of the above mentioned molecules from an antibody, to a growth factor, antisense oligonucleotide, or small molecule agonist or antagonist. Thus if there is a method to rapidly strengthen the definition of the interactions between the molecules involved in the disease process, it should be possible to shorten the development time. During the 1990's, biosensor techniques have become popular methods to make direct measurements of interactions between biomolecules. Biosensors are devices which in some way convert a physical parameter of a biological interaction into a signal. In order to achieve the maximum amount of flexibility, it is preferable to have a user-defined biosensor so that almost any kind of interaction can be monitored. The optical biosensors are the most versatile commercially available systems. This review will cover the design of the leading optical biosensor and its applications in the field of drug discovery.

Experimental Principles - One of the commercially available instruments utilizes continuous flow to monitor interactions between two molecules of biological interest. One molecule (termed the ligand) is immobilized to the sensor surface, and the other interactant (termed the analyte) flows over that surface continuously in a micro-flow cell. As the analyte binds to the immobilized ligand, the resonance angle changes due to refractive index changes and a response is plotted (*vida infra).* It is important to keep in mind that the molecules do not need to be labeled in any way, nor do they need to possess any intrinsically measurable properties. The total run times of an experiment are relatively short (6-10min), and the data is obtained in real-time instead of an end-point assay. The assays may also be automated so that data can be obtained overnight.

Surface Plasmon Resonance as a Detection Technique - It is most desirable to be able to monitor interactions in real time. Biacore AB in Uppsala, Sweden marketed its first real time biomolecular interaction analysis (BIA) instrument in late 1990. These instruments use a detection technique called surface plasmon resonance (SPR) which is based on a physical phenomenon called total internal reflection. Total internal reflectance occurs when light is incident beyond a critical angle upon a refractive index barrier from high refractive index to low. Even though the incident light is reflected, there is an electromagnetic component called the evanescent wave which propagates into the low refractive index media. If the interface between the refractive index barrier is coated with a thin layer of metal, the intensity of the reflected light is reduced at a specific incident angle producing a shadow at the detector. The angle at

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which the shadow is observed is dependent upon many factors including the refractive index of the medium that the evanescent wave is propagating through. Thus the detector is responding to the concentration of solutes in the solution at the surface of the sensor(1). The next question is, how do we get our molecules of biological interest to the surface where this evanescent wave can be detected?

Properties of the Sensor Chip - The flow based instrument utilizes a sensor chip technology for monitoring molecular interactions. There are certain features of this chip that are absolutely necessary: (1) it must be maximally flexible so the interaction system is user defined; (2) it should have a simple, reproducible and rapid method of immobilizing one reactant; and (3) it should be extremely stable and resistant to regeneration conditions. The metal surface (gold) is covered with a matrix that is made up of linear, non-crosslinked carboxymethylated dextran. This matrix allows one to immobilize a molecule to the surface covalently through amine, aldehyde, or thiol chemistry; or one can use a capturing strategy such as streptavidin/biotin(2-4). Additionally, there are surfaces available for capturing 6-His tagged fusion proteins and hydrophobic moieties. After one reactant is immobilized on the chip surface either covalently or via a capture method, the second reactant, the analyte, is passed over the surface at a constant flow rate and the signal is monitored as the injection proceeds. With the proper regeneration conditions, a surface can be used for more than 100 analyses.

Integrated Micro-fluidics - The integrated fluidics cartridge is made up of sample loops, flow channels, and pneumatic valves that are controlled by the computer processing unit and deliver the analyte to the sensor surface. The flow channels are designed for efficient mass transport and low sample dispersion. The advantages of having the computer controlled continuous flow technology are the rapid exchange of buffer and sample, and the reproducibility, precision, and accuracy of the sample delivery. The flow cells themselves have a volume of 60nl and the average flow rate is 10 microliters per minute resulting in an average sample size of approximately 50-70 microliters.

The technology incorporated into biomolecular interaction analysis (BIA) is ideal for moving forward efforts in drug discovery because one can obtain large amounts of functional data about molecular interactions that cannot be obtained in any other way. Because these biosensors are user-defined and thus extremely flexible, they can be used in all facets of a drug discovery program from target identification, through characterization, structure-function analysis, lead identification, and even in clinical trial monitoring. The targets need not be limited to proteins, but can be oligonucleotides, sugars, peptides or larger molecules like vesicles, viruses, or cells. The detection technique and sensor surface are so generally designed that almost any program can use the instrumentation to further their research goals.

Biosensors for Ligand Fishing - There is an increasing emphasis on determining which proteins are involved in a disease process, characterizing those proteins fully, and then finding ways to influence those proteins so as to minimize or remove the disease. There have been huge advances in genomics that have lead to the discovery of new proteins involved in disease processes. It is necessary to find the binding partners of these newly discovered proteins in order to determine how best to fight the disease. BIA technology is very powerful in that one can detect specific binding interactions using very complex mixtures such as crude cell lysates and tissue homogenates as the analyte.

The sensitivity of a BIACORE is generally in the ng/ml range; however, many expressed proteins are in pg/ml concentrations. Thus one usually concentrates solutions prior to applying them to the sensor surface. Once the samples have been passed over the specific surface(s), it is necessary to confirm the specificity of the interaction. This can often be achieved by incubating the sample with a large molar excess of a soluble form of the fishing reagent that is immobilized. If the response obtained previously is specific, the signal should be diminished or removed. There have been many examples of successful ligand fishing experiments using BIACORE technology over the past several years (5, 6).

Table 1 (printed with permission from *BIAjournal*, special issue, 1997)

Receptor	Ligand	Source	Function	Reference
ECK	B61	HCT-8, SK-BR-3	?	(7)
Tyro3	Protein S	FBS, ABAE	anti-coagulant	(8)
Axl	Gas6	ABAE	?	(8)
HEK	AL-1	HPCM	?	(9)
HTK	HTKL	C-1, KATO-III	?	(6)
TIE2	angio-poietin-1	SHEP, C2C12ras	endothelial development	(10)
Flt4	murine Flt4	BNL 1NG A.2	angio-genesis?	(11)

In many of the examples referenced, the proteins that were found as ligands were previously sequenced; however, in at least one case a new protein was identified that had not been previously sequenced (10).

Interestingly, it is getting easier to couple biosensor technology to other technologies such as mass spectroscopy for sequencing and identification of proteins(12-17). The sensitivity of mass spectral analysis enables the researcher to run a sample directly from the biosensor saving the time of transferring the assay to an affinity column. Additionally it is not unusual to find a situation where the biosensor experiment works nicely to find a ligand, but once the fishing reagent is immobilized to a resin, it is no longer active to find its partner in the crude mixture. Therefore biosensors can be a very valuable tool for the target discovery process from the identification stage all the way through to the characterization of the purified protein.

Target Characterization - Once a target is identified, it is necessary to fully characterize the interactions that are the focus. An elegant example of work being done to unravel the mysteries of a complicated system is seen in the delineation of the interactions between estrogen, the estrogen receptor, and its target DNA. The estrogen receptor is a member of a large family of ligand-inducible transcription factors. The steroid hormones as a group are involved in developmental and physiological processes through their ability to perform intracellular communication by binding to their receptors. The receptors are transcription factors that can modulate the expression of target genes. Members of this family of receptors have structural and functional similarity, yet they bind to very specific target sequences of DNA.

It was hypothesized by one group that the hormone binding to receptor may effect the kinetics of the interaction of the receptor with DNA without effecting its

overall affinity. This is a difficult hypothesis to prove using conventional methods; however, the optical biosensor provided the flexibility and detection capabilities that they needed (18). Experiments were performed by immobilizing on the sensor surface the specific and control DNA target sequences. It was first confirmed that the estrogen receptor in the presence of hormone bound specifically to the target DNA sequence and not to the control surfaces. The kinetics of the interaction of the receptor with DNA in the presence of various hormone ligands or other synthetic agonists or antagonists was observed. It was concluded that the overall affinity of the receptor/DNA complex changed little (at most by a factor of 5); however, the kinetics of the interactions changed drastically. The presence of an agonist induces rapid formation of an unstable complex with DNA. On the other hand, binding of an antagonist induces a slow formation of a stable receptor/DNA complex. This correlates nicely with the fact that binding of agonist accelerates estrogen receptor turnover by 1000 fold over the antagonist's effect. Thus these authors are suggesting a correlation between the rate of gene transcription and the frequency of complex formation. There have also been studies on other steroid hormone receptors along the same lines (19-21).

Another use for biosensors is being found in the realm of phage display library screening. It is often desirable to construct a ligand for a target protein that will have a higher affinity than the natural ligand. It is becoming more popular to perform these types of studies using phage display libraries. One group in particular has shown that optical biosensors can be extremely useful in selecting high affinity ligands on phage (22-25). The early work generally used several different techniques for the selection process and ended with confirmation of high affinity phage isolates through biosensor screening. Recently, however, the phage were screened directly on the biosensor in order to isolate the highest affinity clones (24). In this example, the investigators were searching for the highest affinity single chain antibody to a particular antigen for therapeutic use. The unique capability that the biosensor technique offers is the ability to control the amount of antigen immobilized for selection during *in vitro* affinity maturation. This is important because it allows for the accurate concentration measurement of the specific phage in solution. They determined that the concentration values that they determined correlated very closely with positives observed in an enzyme linked immunosorbant assay (ELISA). As part of the biosensor assay, one must regenerate the antigen surface by stripping off the bound phage. There are many different ways to do this using changes in ionic strength or pH. The investigators found that they were able to screen for higher affinity clones simply by using different regeneration solutions. As they moved from weak to strong regeneration solutions they were selecting for the highest affinity clones. From these studies they realized that it is likely that many people who elute bound phage from resins may be choosing inadequate washing solutions to remove the highest affinity moieties and thus may be selecting and amplifying lower affinity clones.

Biosensors in Screening - The current design of commercially available optical biosensors does not lend itself to high throughput screening. With careful planning and assay design it is possible to achieve capabilities of 2000 compounds per day; however, this is more the exception than the rule. The biosensors can be extremely useful though in designing a high throughput primary assay and then confirming any hits through a secondary screen using the biosensor. One group has been using their biosensor for just those purposes. This group is focusing on *Met* as a target for anticancer therapies (26). *Met* is the hepatocyte growth factor receptor which belongs

to a family of tyrosine kinase receptors. It has a distinct two-tyrosine docking supersite in its sequence which appears to be solely responsible for mediating virtually all of the downstream effects of receptor activation. Malignancies appear to result from overexpression or constitutive ligand-independent activation of the kinase. Experiments had shown that mutation of the docking site removed transforming ability. Therefore these scientists wished to search for inhibitors that would block the interaction of the docking site with its natural ligands.
They began by characterizing the interaction between the docking site peptide and the full length ligand and truncated ligand. It was quickly determined that the truncated ligand was sufficient to give accurate results. Based on the biosensor data, a high throughput SPA assay was designed. Initially the SPA results were validated against the biosensor data until there was confidence in the radioactive assay. One hundred thousand compounds were then rapidly screened and there were 246 positives that resulted. One of the disadvantages of SPA and other high throughput screens is that they have a high false positive hit rate. Using the biosensor, these investigators were able to quickly determine that 35 (of the 246) molecules showed an inhibitory activity at concentrations below 50 micromolar. Those molecules that showed the most promise, were then studied by directly immobilizing the truncated ligand and flowing the inhibitor in solution. The investigators found that they could get kinetic rate constants for some of these molecules eventhough their molecular weights were between 300 and 600 daltons. They are in the process of validating these assays. Thus the biosensor was used effectively to create a successful high throughput assay in a minimum amount of time, and it provided a swift and accurate way of screening hits from the primary assay for confirmation of activity.

Biosensors in Validated Assays - Once the basic research has been accomplished on a target and a lead has made it through *in vitro* studies, it then can be moved into pre-clinical and clinical trials if it looks promising enough. For protein therapeutics it is vital to monitor antibody production to the therapeutic agent by the host. This is normally done by ELISA assays and can be time consuming. It also does not provide any information as to the isotype of the antibody being produced which can be very important to the scientists performing the trials. One group has validated an assay using a BIACORE 2000 that can quantitate the amount of humanized antibody in patient serum and can also detect whether there are any antibodies directed against this therapeutic agent and isotype those antibodies if present.(27) It was important for these investigators that this assay can utilize one injection of diluted serum such that the volume needed was as little as 10 microliters of patient serum.

There are many factors that must be considered when validating an immunoassay. The accuracy and precision must be validated, in addition one must worry about the stability of the analyte in the sample, different sources of the sample, and the effects of freeze-thaw cycles on the analyte. Finally the curve fitting and standard curves must be appropriate. There are a few factors that are unique to a biosensor assay which include the number of regeneration cycles, stability of the immobilized ligand and binding capacity of the immobilized ligand. Factors relating to monitoring of patient response to an anti-IL-5 humanized monoclonal antibody have been described (27). The concentration of the humanized monoclonal was measured by passing the serum sample first over a flowcell with IL-5 immobilized, then that same sample went over a serially connected flowcell with the humanized monoclonal in order to monitor for antibody production by the patient. This assay worked exceptionally

well with excellent precision and specificity and used less patient sample and took less time than conventional plate immunoassays.

Conclusions - Optical biosensors have been commercially available since 1990. It is clear that in the last two years the use of these biosensors has expanded exponentially as researchers have realized their potential in monitoring molecular interactions in a label-free and real-time manner. In 1997 alone their were over 200 scientific papers published using these devices. There have been advances in the technology that have allowed for detection of molecules as small as 200 daltons. Additionally, interactions can be pulled out of very complex mixtures which cuts out purification steps and saves time. The specificity and reproducibility of the data obtained allows researchers to have greater faith in the data they obtain. Biosensors also have a low incidence of false positives during screening because of the specific nature of the surface employed for the experiments. It appears at this time there will be areas of research where these instruments will not be useful as a primary or secondary means of obtaining vital interaction data.

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Chapter 30. Current and Future Trends in High Throughput Screening for Drug Discovery

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Introduction - In the 1970's a revolution began in the pharmaceutical industry with the technological advances made in molecular biology. Prior to this, the molecular targets that were used to discover new drugs had to be purified from the natural source - a long and labor intensive process. With the advances in molecular biology, target quantity and purification were no longer a bottleneck in the search for new drugs. More recently, this revolution has continued with advances in combinatorial chemistry (1-5). These advances, which began in the 1980's and have continued through today, have made it possible for chemists to generate hundreds of thousands of potential therapeutic compounds in the same time that it used to take to make several compounds. Couple these two major advances with the current advances in genomics (6-7) and the potential exists for even greater changes in the way in which compounds are discovered for the treatment of disease.

Advances in molecular biology, combinatorial chemistry, and genomics have revolutionized the way in which we search for new drugs. The screening technologies of the past decade cannot sustain the increased number of both targets and compounds that need testing. Consequently, new screening technologies are currently under development that should help to overcome this current bottleneck in drug discovery. Any advanced screening technology under development, however, must alleviate several critical problems that currently plague the field.

First, certain molecular targets are very difficult to produce in the quantities needed for current screening capabilities in a cost effective manner. Some targets must still be purified from a natural source because target expression is not possible in hosts such as *E. coli* , *S. cerevisiae*, or baculovirus. Even when a target can be expressed in a standard host, the increased number of compounds available for testing from combinatorial and medicinal chemistry necessitate very large scale protein production in order to have enough of the target for comprehensive library screening. If a screen could be miniaturized from 100 to 1000-fold, then the amount of protein needed for the screen could be reduced from hundreds of milligrams or grams down to several milligrams. A conversion, in essence, from the fermenter scale down to the analytical scale.

Second, the quantities of compound available for testing from combinatorial libraries are exceedingly small, usually on the order of several hundred picomoles from a single combinatorial bead or several milligrams from a program in parallel synthesis. The small quantities of compound synthesized in a combinatorial effort make it difficult using current methodologies to screen these compounds at a concentration that is usually considered acceptable in drug discovery. Once again, miniaturization by 100-1000-fold in screening volume could overcome this limitation. In this case, the compound from a single bead in a combinatorial library would be sufficient for several different screens.

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Third, new screening technologies must also be amenable to strategies currently available in drug discovery. For example, over the past several decades, medicinal chemists have synthesized tens of thousands to hundreds of thousands of compounds for therapeutic programs. New screening technologies must be able to take advantage of these discrete compound libraries as a continuing source of diversity. From the biological side, it is important for the new technologies to be compatible with current methods in biological assay development. For example, such techniques as scintillation proximity assays (SPA), cell based reporter gene assays, fluorescence, bioluminescence, chemiluminescence and ELISA type formats must be integrated with the new screening technologies.

The purpose of this chapter is to review the current "state of the art" in leads discovery, the new technologies that should be available in the near term, and the technologies that are likely to be available in the years to come. The chapter will focus on how these current and future technologies are meeting the challenges described above, what the current limitations are with each technology, and the overall likelihood for success.

Current High Throughput Methods of Drug Discovery - The 96-well plate has been the standard format for screening during the past decade (8-9). The vast majority of biological screening assays can be performed in this format and a substantial amount of instrumentation has developed around it. Recently, a number of groups have begun to explore miniaturized formats with an initial move to a 384-well plate (9-10). These two formats have been and will continue to be the workhorses of the drug discovery industry for the near future. They offer a number of advantages over other formats currently available, namely that they can be used in a high-throughput system. Most liquid handling systems and detectors can be fitted with plate stackers that allow for unattended use. Currently available compound decks can be analyzed for compounds with desired biological activity using either of these formats. Total throughput with either of these systems can reach 100,000 discrete tests per week. The analysis of a compound deck against a single target usually takes from several weeks to several months depending upon the complexity of the assay.

Recent developments in liquid handling have dramatically increased the speed with which assays can be run in either the 384-well or 96-well formats. Some currently available systems can process from several hundred to a thousand 96-well plates per hour, depending upon the complexity of reagent addition (11). This translates into approximately 20,000 to 90,000 discrete data points per hour, suggesting that it may be possible to screen entire compound libraries in a single day with existing technologies. Unfortunately, the increased speed provided by the advances in liquid handling are still hampered by the limitations in signal read times. Advances in signal analysis equipment such as spectrophotometers, scintillation counters, or fluorometers, have not kept pace with the advances in liquid handling. Even the most rapid devices require several minutes to move and read a single plate. This limits the number of plates that can be analyzed on any given day to several hundred unless one invests in multiple detectors. Reagent availability also may limit screening in this format.

Compound Pooling Strategies - One method to circumvent the problems just described is by the use of compound pooling strategies (12). This technique was first used out of necessity by labs using the split and pool strategies for making combinatorial libraries (1,13). In most cases, the combinatorial libraries were provided to the biologist as a

pool of closely related compounds for testing against the target of interest. However, this can also be applied to discrete compound libraries available from the work of medicinal chemistry (12). Matrix pooling is the strategy in which, for example, a 10 X 10 matrix of discrete compounds (100 total compounds) is pooled both in the X and Y directions resulting is 20 sets of 10 compounds each. This strategy reduces the library size by a factor of five but also allows each compound to be tested in duplicate since each compound resides in one pool on the X axis and one pool on the Y axis.

The advantages of compound pooling are several fold. First, the speed with which compounds can be analyzed increases in proportion to the pool size. The matrix pooling strategy described above would decrease screening times by about a factor of five. Another advantage to pooling is the savings in biological reagent. Since biological reagents are usually the limiting resource in screening, a matrix pooling strategy would use only a fraction of the reagent that would be consumed in screening discrete compounds.

There are also a number of problems and limitations involved in using a pooling strategy. As was learned early on in combinatorial chemistry, it is usually very difficult to deconvolute a mixture of compounds to discern which one was responsible for the observed activity. The invention of photocleavable linkers and bead encoding strategies has made this process somewhat simpler (3,5,14-16). In this case, only a small amount of material is released from the resin for primary testing. If activity is present in the pool, the beads are separated into a single bead per well and additional compound released for retesting. The compound present on an active bead is then determined via its code. Another strategy is to simply cleave all the material from the resin and simply resynthesize all compounds from any active pool. Both of these strategies are labor intensive and slow.

Another drawback of pooling strategies is the inherent synergistic and antagonistic effects of multiple compounds within any pool. For example, a pool containing 10 discrete compounds, with each component having low biological activity, would appear to have a high activity when tested as a pool due to the summation of 10 weakly active components. Upon deconvolution, however, each individual compound would not appear to be biologically active when tested at the same concentration. The converse is also possible. Other components in the pool may mask an active compound. For example, an active compound could be complexed with an inactive compound thus masking the activity. Thus, the possibility of both false positive and false negative signals is very high with pooling strategies. The pooling strategy is usually only used when speed and/or conservation of reagent are critically important. It should also be noted that the savings in reagents is somewhat mitigated by the need for increased reagent use for retesting.

Gel Diffusion Assays - Gel diffusion assays are another means by which both combinatorial and discrete compound libraries can be analyzed for compounds of interest (17-18). In this format, the biological target is mixed in soft agar and spread as a thin film. A combinatorial bead based library is then spread onto the surface of the agar film. Compound is then released from the library and the compound is allowed to diffuse into the agar. Compounds from discrete compound libraries are pipetted onto the surface of the agar, usually in a small volume of DMSO, and allowed to diffuse into the agar. After allowing for diffusion and potential inhibition of biological activity, an appropriate developer is sprayed onto the surface of the agar. Areas in which the compound present has inhibited biological activity will show up as distinct zones.

This technique is of limited utility because a number of criteria must be met for implementation. The biological reagents must be compatible with the soft agar matrix and the reaction must be detectable either colorometrically or fluorometrically. The reaction time must also be short, usually less than 2 hours, because the test compound is free to diffuse throughout the agar matrix. At some point, the concentration of the test compound will drop below the level of detectability. Another potential problem is that the compound may not dissolve/diffuse from the resin or it may precipitate when placed on the agar film. The major advantage, however, is that for appropriate assays, hundreds of thousands of compounds can be tested in a single day.

Robotics and Workstations - In both chemistry and biology there are continuing discussions regarding the advantages and disadvantages of using either robots or workstations. A number of robotic systems have been developed for use in high-throughput screening (HTS) and for combinatorial synthesis (19,20). The advantages of these systems are that, at least in theory, they are "walk away" devices. That is, once the initial programming and reagents have been loaded, the operator can walk away until the task has been completed. Most of these robotic systems have a throughput of several hundred plates per day. One of the biggest advantages is that practically any device can be interfaced to the system allowing for virtually any configuration and assay format. On robotic systems, each plate is handled in a sequential manner versus the batch mode that is usual for workstations. That is, on a robotic system, each plate is taken sequentially and individually through the entire process. This method limits the number of plates that can be processed in any given time. This limitation has been partially overcome with the use of scheduling software that allows multiple sequential events to be run in parallel.

There are several major disadvantages with these large robotic systems (21-23). First, these systems tend to be costly, but, more importantly, they tend to take up significant amounts of precious lab space. Typically, a robotic system can occupy an entire lab that could potentially hold several workstations. Another major limitation to this system is that if the robot itself fails, it will take all the peripheral equipment off-line with it. As a corollary, failures in any peripheral equipment may also affect the entire system. This is especially true if the failure occurs when the operator is not present. A failure early in a given run could cause a failure of the entire run if the operator is not contacted or is not capable of restoring the system. This can result in the loss of a significant amount of reagent. It is also important to note that with robotic systems, only a single assay can be performed at any given time. Like plate handling, targets must be assayed sequentially.

Additionally, while the theoretical throughput of a robotic system can approach 100-150 plates per day, the relative sluggishness of the transport process requires that critical reagents be stable for extended times (23). This limits the type of assays that can be performed. These systems also tend to have rather large "dead" volumes for reagents. For reagents that are limited in availability or stability this represents a significant limitation to the system. Finally, the time needed for installation of new devices on an integrated robotics platform often exceeds the time allowed for completion of an assay. This is usually due to the complexity of I/O communications and system reconfiguration.

Workstations are highly specialized stand-alone devices where a human acts as both as the robotic arm and is present for critical oversight (22). Workstations

include devices such as liquid handlers, detectors, etc. These systems are very flexible because devices can be run in series or parallel depending upon need . For example, several liquid handlers can be used in series with several detectors used in parallel. The human operator is responsible for moving stacks of plates from one device to another. This oversight plays a critical role in that errors can be rapidly detected and corrected. Because each workstation performs a highly specialized function, throughput tends to be significantly higher than with robotic systems. The ability to perform assays in batch mode contributes significantly to the overall speed of the system. Unlike robotic systems in which a failure in a detector would impair the entire system, a system using independent workstations would not be affected because another detector could be easily substituted.

The human interface with workstations is both an advantage as well as a disadvantage. The advantage is that the human can decide immediately if the assay/device is performing as expected and, if not, can take immediate corrective action. The disadvantage is that for particularly tedious processes, humans tend to commit more errors than a robot. As a general rule, robots tend to be used for assays that are very simple, especially one step processes, or for multi-step processes when the reagents are very stable. Workstations, however, are usually used for pure speed and throughput regardless of the number of steps or reagent stability.

Data Management - With the large amounts of data that can be generated with currently available screening systems, data management is crucial. Links within a database between compound code, structure, activity, specificity, purity, solubility, serum protein binding, serum half-life, and bioavailability are necessary in order for the chemist to choose the one or several structures on which to begin SAR. The use of a relational database is necessary in order to access all of this data in a reasonable fashion. A relational database allows related data to be grouped together in a single database table. Consistency of data entry is enforced through the use of defined database fields. Consequently, searching and analyzing across database sets is greatly simplified. For example, if a number of lead compounds are discovered that interact with Target A, a simple search will reveal if these compounds have been shown to interact with any other targets that may have been screened in the past. More importantly, a relational database also eliminates the tedious management of files usually associated with the use of spreadsheets. These relational databases have been used for a number of years in other data-intensive industries but have just recently been implemented in HTS data management.

Near Term Trends - Plates with 96 and 384 wells will continue to be used in the foreseeable future. Their utility in primary screening will give way over the next 1-2 years to new miniaturized technologies. The first steps in assay miniaturization will most likely be a move to 1536-well (2-5ul volume) and 6144-well (0.2-0.9ul) plates over the next 1-2 years followed by a move to microchip arrays in the next 7-10 years.

Higher density well arrays are currently under development by a number of different groups. Several groups have taken what has been termed the evolutionary approach in that they are slowly moving from the standard 96-well plates to 384-well plates then to 864-well plates and finally to much higher density plates such as the 1536, 3456, or 6144 (9600). All of these plates are designed based on the Society of Biomolecular Screening standard footprint and dimensions. The advantages to this evolutionary approach are several fold. This sequential approach nearly guarantees success at every step because each step is small and incremental. For example, the

step from a 96-well plate to a 384-well plate is a difference in volume from 100 ul down to approximately 50ul. The difference from the 384-well plate to a 864-well plate is from 50ul down to 20ul. In theory, each system is well defined, and it is relatively simple to decrease the total reaction volume by a factor of 2 to 2.5-fold. Most current instrumentation, at least with minor modifications, will accommodate the 384-well plates but new instrumentation must be developed for the higher density arrays.

The advantages of the evolutionary approach need to be tempered by the minimal savings in reagent and time at any individual step. Screening in a 384-well plate requires only 50% of the total reagent needed for the 96-well format, plate cost is approximately the same, waste stream is somewhat reduced, and screening time is not significantly changed. Cost of modifying equipment in order to accommodate each new format may be significant and each step requires new modifications. Overall, the cost of implementing the sequential move may not offset the small gains made. It may be more feasible to move from a 96-well standard format to a 96-well quarter volume system. The 96-well quarter volume system gains all of the advantages of the 384-well system without new expenditures for updating equipment.

Another approach taken by several groups has been termed the revolutionary approach in that it moves immediately from the standard 96-well format to a much higher density such as the 1536, 3456, 6144, or 9600 (24-27). In these assay formats, the well volume decreases by a factor of from 10-1000-fold. Consequently, both the reagent needed to perform the assay in these formats and the signal generated are reduced by an equivalent amount. Because reagent supply is often the limiting factor in HTS, a move to a much higher density, low volume array is very attractive. This is true not only for biological reagents but also for chemical compounds as well. In combinatorial chemistry the amount of compound synthesized on any given bead is on the order of 100-200 picomoles. If all of this material were released into a standard 96-well screen, the effective screening concentration would be from 1-2 micromolar. This is lower than the usual screening concentration of from 5-10 micromolar. If all of the material is released into the assay for testing at as high a concentration as possible, none remains for retests or for analytical analysis. Consequently, the only way to confirm activity is to resynthesize the compound - partially defeating the purpose of combinatorial bead-based synthesis.

This problem does not occur with the higher density, low volume plates. The 9600-well plates have a well volume of approximately 0.2ul (26). Release of as little as 2 picomoles of compound from a bead is sufficient to achieve an effective concentration of 10 micromolar in the well. This leaves the majority of the material still bound to the bead and available for retests or for analytical analysis. In theory, an individual bead could be used in up to 50 different tests, but, in practice, this is probably not practical.

Challenges of Miniaturization - Unfortunately, while these higher density arrays show great promise, the instrumentation for implementing these systems is just now becoming available. Instrumentation such as spectrophotometers, fluorometers, and scintillation counters that rely on photomultiplier tube technology are not amenable for use with these higher density formats. A photomultiplier tube can be columnated so that it can measure signal from a 384-well plate but further reduction in size is not practical. With these vast arrays of wells, it is also not practical to analyze each well individually. Recent advances in CCD technology and laser scanners have made it possible to image entire areas, such as the surface of a microwell plate, with sensitivities that approach that observed with photomultiplier tubes (26). In order for

CCD technology to be accepted as a means of signal analysis, the system needs to have a high degree of spatial resolution because the wells are very small, less than 1.5mm square and 1.5mm center to center spacing, and have good low light capture capabilities. Since the well volumes are from 100 to 1000-fold less than in a standard 96-well format, the signal emanating from any well will be proportionately less and more difficult to capture. Conventional lenses are problematic for these purposes because they usually display some degree of spherical aberration that tends to distort the image as it moves further from the center of the lens. Systems are currently available that overcome most of these problems. These systems usually contain a high resolution CCD camera, 1K X 1K chip, and a large telecentric lens. Using this CCD technology, images from antimicrobial screens and matrix metalloproteinase screens have been prepared (26).

Liquid handling also poses a challenge since the volumes in these miniaturized formats varies from 0.2 ul to 7 ul per well depending upon the well density. These microvolume wells require the addition of nanoliter volumes of reagent. Conventional liquid handling devices are not amenable for this task because they are not typically accurate below 1 ul due to surface tension effects. Liquid handling systems based either on ink jet printer technology or piezoelastic technology are capable of accurately dispensing nanoliter volumes (28-30). Other systems for both image detection and liquid handling may become commercially available within the next year.

Two inherent problems with assay miniaturization both involve surface area to volume ratios. Upon well miniaturization, the surface area to volume ratio increases dramatically. The relative increase in surface area compared to total volume leads to a dramatic rise in the evaporation rate from the well. Because the wells contain microvolumes, evaporation can be a severe problem. It is not practical to maintain electronic equipment at very high humidity so both the liquid handling steps and imaging must be performed as rapidly as possible. The second surface area to volume ratio involves the plastic surface area that comes in contact with the reaction. Because the total plastic surface area is very large in comparison to the reaction volume, any interaction between the reaction components and the plastic will be magnified. This can be particularly troubling if the protein being tested adsorbs onto the plastic surface and denatures or loses activity. The methods and instrumentation used to overcome these problems have recently been described (26).

The advantages of miniaturization to these higher density formats are several fold. In a number of cases, obtaining the biological target in sufficient quantity for screening in a 96-well or 384-well format is not possible. This is especially true if the target cannot be made recombinantly and has to be purified from a natural source. In this case, a low density, high volume format (96-well) limits the number of compounds that can be assayed whereas the high density, low volume format (e.g. 9600-well) allows screening of all compounds in the library. Since 100 to 1000-fold less reagent is needed to complete an entire screen, time that would have been needed to purify large quantities of the original target can be spent cloning and expressing new targets thus increasing the overall chance of successfully finding new drugs. Similar savings in chemical test compounds also have been observed. In the case of combinatorial bead based libraries, miniaturization may make single bead screening possible. By miniaturizing the assay, larger combinatorial libraries or higher copy numbers within libraries may also be possible because the libraries can be made on much smaller resin. A 10 um diameter resin bead contains more than sufficient compound to achieve screening concentrations in the 10uM range with sufficient compound remaining for

secondary testing and mass spectroscopy analysis. Because the volume of a bead varies as the cube of its radius, a library on 10um resin will contain nearly 1000 times as many copies of each compound as the same library on 100um resin. Obviously this could extend the life of any given library.

These miniaturized systems suffer from one major drawback, namely that they have been introduced so recently that the breadth and scope of biological reactions amenable to this format have not yet been fully determined. Recent publications indicate that for a 9600-well (0.2ul) assay system, antimicrobial (both fungi and bacteria) assays can be performed (26). Soluble enzyme assays that are linked to light output such as fluorescence, bioluminescence, or chemiluminescence can also be performed in this format. Certain assay types such as receptor binding assays, mammalian cell reporter gene systems, scintillation proximity assays, and kinase assays may not be easily formatted into a 0.2ul volume but may be formatted into the somewhat lower density plates such as the 1536-well (5-7ul) or the 3456-well (1ul) plates.

These miniaturized systems have met two important criteria, namely that they can be used to test compounds from both traditional medicinal chemistry and from combinatorial bead based libraries. Currently cell based assays utilizing reporter genes or soluble enzyme assays can be performed in this format. Receptor binding assays, kinase assays, and SPA assays need considerable development. Full acceptance of this miniaturized format will occur only when these final technical hurdles are overcome and the instrumentation needed to perform them are widely available.

Material Science and Agriculture - Combinatorial chemistry is also having a major effect on the fields of material science and agriculture. Both these fields are engaged in assay development for their respective purposes. In agriculture, novel compounds that can specifically inhibit plant germination or growth, act as antifungals, or as insecticides are widely sought. Many current methods for discovering these compounds involve the use of whole plants or insects. The move in agriculture to molecular based targets is just beginning, and as such, this is a rapidly developing area for the use of miniaturized screening technology. The potential arises for this field to move directly to miniaturized formats, forgoing 96-well screening altogether.

Material science is taking a similar approach to discovering novel materials through combinatorial chemistry. Several groups have used combinatorial chemistry to look for new catalysts or for new superconducting materials (31-33). The development of this field with respect to assay development may parallel the advances made in the pharmaceutical industry. The advances made with regard to assay and screening technologies in either agriculture or materials science may be applicable to drug discovery.

What the Future Holds - Microreaction technology is still in its infancy with the number of reports in peer-reviewed journals still very limited. A number of groups have recently reported the potential for both combinatorial synthesis and target screening on microchips (34-39). The microfabrication techniques used to manufacture these microchips have been lifted directly from the computer industry. These techniques allow for the production of a variety of inexpensive, three-dimensional microstructures that can be of nearly any design and produced from nearly any material. Micropumps, micro heat exchangers, micromixers, microfilters, microextraction modules, and microdispensers are all examples of devices that have been constructed in a microchip

format (37). In these systems, microchannels controlled by miniature valves allow reagents to flow into microreactors. These microreactors usually contain on the order of several hundred picoliter volumes. The micro channels and reactors are prepared via standard photolithography in a suitable material such as a silicon wafer. These chips are approximately the size of a 35mm slide and contain intricate networks of channels and reaction vessels. Liquid is moved through the channels using electrokinetic means. Assays in as little as 200 picoliters could potentially be performed using this new technology.

A microchip device to perform micro-scale enzyme assays has recently been reported (38). Using this device, precise nanoliter volumes of enzyme, substrate, and inhibitor were mixed using electrokinetic flow. This type of device regulates reagent dilution and mixing through the applied electric potential across the terminus of the channel. On this volume scale, mixing occurs only via diffusion. Using beta-galactosidase as a model system, they were able to determine Km and kcat values that were in good agreement with those obtained in a standard 96-well format. Importantly, the amount of enzyme and substrate used for the entire reaction was 4 orders of magnitude less than would have been consumed in a conventional assay. For this system, each individual assay required only 120 picograms of enzyme and 7.5 nanograms of substrate.

Because most of the systems that have been currently described rely on silicon wafers for the production of these microchips, there are some obvious and inherent problems that must be overcome before this technology shows wide utility for high throughput screening. As with any miniaturization technology, as the volume is decreased, the surface area to volume ratio becomes much larger, and, consequently, the surface area that can interact with the reaction components becomes much larger. The use of silicon wafers may exacerbate this problem as it has been well established that many proteins adsorb onto glass. It should also be noted that reaction components other than the protein could also interact adversely with the chip material thus yielding false positive or false negative signals. However, because the channels and reaction vessels are very small, it may be relatively simple to coat the entire surface with the test protein or other material thereby blocking deleterious interactions. Because the amount of protein used in these reactions is so low, the use of additional protein to block the surface will not contribute significantly to the overall protein usage. Other potential problems include applicability to of cell based assays, the addition of bulk reagents such as enzyme and/or substrate to the microchip, the addition of compound library to the chip, and the blocking of potential interactions between the chip and the reaction components. This technology shows promise, but significant hurdles still must be overcome.

Moving the Bottleneck - The recent advances in combinatorial chemistry and high-throughput screening are having a ripple effect throughout the pharmaceutical industry. Combinatorial chemistry is providing hundreds of thousands to millions of potentially interesting new molecules, and high throughput screening allows additional targets to be analyzed much more rapidly and economically. The widespread use of these two new technologies has caused a bottleneck in downstream analysis. For example, a million compound library may provide 10-fold more "lead" compounds than a hundred thousand library. How then can the best lead structures for SAR be chosen from the increasing number of leads? Additional information must be ascertained on both the primary leads as well as any compounds that are synthesized during SAR analysis. In essence, what has traditionally been considered "drug development", *i.e.*,

bioavailability, serum protein binding, and compound solubility, now needs to be performed much earlier in the discovery process. High-throughput screening groups in collaboration with the Development groups have the expertise to develop high throughput versions of bioavailability, serum protein binding, and compound solubility assays.

With additional information on both the primary and secondary compounds, chemists should be able to rapidly determine which of all the possible compounds have the desired biological activity that will be most easily turned into a drug. This should, in turn, ease the bottleneck on "development" because fewer compounds will fail during that process. This new strategy for drug discovery, however, relies upon close cooperation between the high-throughput screening group, combinatorial and medicinal chemistry groups, and the drug development group.

The future of drug development is never certain, and new technologies must be constantly evaluated for both their potential merit and limitations. Each advance in a particular area leads to a bottleneck somewhere else in the drug discovery process. No one technology will solve all of the current challenges in drug discovery, but, if current trends continue there will certainly be a number of new technologies from which to choose.

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Chapter 31. Second Generation Antisense Oligonucleotides: 2′-Modifications

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Introduction - The use of synthetic oligonucleotide ligands, designed to sequence-specifically bind by Watson-Crick base pair recognition to RNA receptors, is a relatively new drug discovery paradigm. This approach, referred to as antisense technology, began to receive considerable drug discovery efforts about nine years ago, primarily by several new biotechnology companies. Phosphorothioate oligodeoxynucleotides (PSs), developed as first generation antisense agents, have been shown to operate by this novel drug mechanism and several PSs have progressed to late stage clinical development. Considerable knowledge has been amassed concerning the pharmacology, pharmacokinetic and toxicology properties of these first generation antisense oligonucleotides, and along with this, certain limitations of PSs as antisense agents have emerged. Structural changes are required to overcome these limitations and to continually improve this novel oligonucleotide drug class.

Although many types of modifications, at essentially all available positions that do not directly interfere with Watson-Crick base pair recognition, have been examined, only 2′-modifications have demonstrated sufficiently interesting drug properties to be moved into advanced studies (1). Indeed, the first, second generation modified oligonucleotides, 2′-*O*-methy (2) and 2′-*O*-methoxyethyl (3) gapmers have just recently entered clinical trials for CMV retinitis in AIDS patients.

This chapter will briefly discuss the advantages and limitations of the phosphorothioate oligonucleotides as antisense drug candidates. It will then briefly summerize the 2′-modified class of oligonucleotide that address PS limitations. This particular class of second generation modifications, on the basis of impressive, positive pharmacology, pharmacokinetics, and toxicity data in animals, is expected to eventually replace first generation PS antisense oligonucleotides as drug candidates.

A number of general reviews, primarily focusing on antisense oligonucleotide medicinal chemistry have periodically been published (1). In particular, three *Annual Reports in Medicinal Chemistry* have specifically discussed antisense technology (4-6).

ANTISENSE OLIGONUCLEOTIDES IN CLINICAL TRIALS

Approximately 16 oligonucleotides with antiviral, anticancer, anti-inflammatory and restenosis indications have entered into human clinical trials (Table 1). These are all full length phosphorothioates ranging from 15 to 25 bases. Fomivirsen, ISIS 2922, a 21-mer PS, targets an immediate early mRNA in region 2 of cytomegalovirus and inhibits expression of two regulatory proteins (7). It is given locally by intravitreal injections for cytomegalovirus-induced retinitis in AIDS patients. It has the distinction of the first oligonucleotide to exhibit positive response from local administration. Fomivirsen, the most advanced PS drug candidate, was found to be safe and efficacious in a pivotal Phase III clinical trial (8) and has been submitted for approval (NDA). ISIS 2302, a 20-mer PS targeting intracellular adhesion molecule one (ICAM-1) (9) is in Phase II anti-inflammatory trials for five indications. ISIS 2302 recently has demonstrated very exciting activity against Crohn's disease (10). It has the distinction of the first oligonucleotide to exhibit systemic activity in humans. The early clinical trials of ISIS-2105 for genital warts (11) and Hybridon's GEM-91 (12) for HIV have been terminated due to lack of activity in Phase I/II.

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LIMITATIONS OF FIRST GENERATION PHOSPHOROTHIOATES

Pharmacodynamic Limitations - For every thiophosphate linkage in a phosphorothioate oligonucleotide, a destabilization of the heteroduplex with complementary RNA of approximately - 0.7° C occurs, relative to parent DNA (16). This amounts to approximately 14° C destabilization in a 21 mer phosphorothioate, or approximately 3-4 orders of magnitude decrease in K_d compared to unmodified DNA hybridized to RNA (17-19). Thus, PSs are not likely to invade dsRNA structures and they must be targeted to accessible single-stranded sites. This low binding affinity per monomer unit of a PS limits the sites on mRNAs that they can effectively targeted. Even when PSs are targeted to single-stranded RNA and supposedly bind sequence specifically, they are not likely to elicit biological activity simply because of this binding. The consensus mode of action of antisense PSs is that on sequence-specific binding to target RNA, an endogenous, ubiquitous endonucleolytic enzyme, RNase H, binds the duplex and cleaves the RNA strand of the heteroduplex. Therefore, currently, antisense technology requires a RNase H mechanism which in turn requires a contiguous stretch of PSs in the backbone. This is in the form of thiophosphates which provide resistance to degradation by nucleases. At higher concentrations, PSs bind to RNase H and may prevent its serving as an effector of biological activity.

Table 1. Antisense Oligonucleotides Which Have Started Clinical Development

Oligonucleotide	Molecular Target	Disease Indications	Status	Sponsor	Ref. #'s
ISIS 2105	HPV 6 and 11 E2 gene product	Genital warts	Terminated 12/28/95	Isis	(11)
ISIS 2922	HCMV IE gene product	CMV retinitis	NDA Filed 4/09/98	Isis/Ciba Vision	(3,8)
ISIS 2302	Intracellular Adhesion Molecule-1 (ICAM-1)	Renal allograft, rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis	II/III, Crohn's II, Others	Isis/ Boehringer Ingleheim	(3,9,10)
ISIS 3521/CPG 64128A	Protein kinase C-α	Cancer	II	Isis/ Novartis	(3)
ISIS 5132/CPG 69846A	C-*raf* Kinase	Cancer	II	Isis/ Novartis	(3)
ISIS 13312	HCMV 1E gene product	CMV retinitis	I/II	Isis	(3)
ISIS 2503	Ha-*ras*	Cancer	I	Isis	
GEM-91	Gag gene	HIV	Terminated 7/25/97	Hybridon	(12)
GEM-92	Gag gene	HIV	I	Hybridon	(2)

GEM-132	UL36 & UL37	CMV retinitis	I	Hybridon	(2)
GEM-231	Protein kinase A	Cancer	I	Hybridon	(2)
LR-3280	C-*myc*	Restenosis	II	Lynx/Inex	(13)
LR-3001	C-*myb*	Cancer	I	Lynx/Inex	(13)
G3139	Bcl-2	Cancer	I	Genta	(14)
GPI-2A	Gag gene	HIV	I	Novopharm Biotech	(15)
	C-*myb*	Leukaemias	I	Univ. Penn. School of Med.	(16)

Pharmacokinetic Limitations - Significant oral bioavailability of PSs has not been reported. This may be the most serious shortcoming of oligonucleotides as therapeutic agents; however, recent studies of phosphorothioates stabilized by 2′-*O*-methyl modifications at the 3′- and 5′- flanks exhibit encouraging results (20,21). Also, penetration of the blood brain barrier has not been achieved for phosphorothioates. The pharmacokinetics of PSs which are relatively large, negatively charged molecules, are dependent on the administered dose. This is suggested because of the propensity of PSs to bind to serum proteins (22). Saturation of protein binding sites may occur and cause variation in PS absorption and distribution. Modifications of PSs to alter the pharmacokinetic properties remain an important medicinal chemistry problem. A clear understanding of how PSs are taken into cells, absorbed by various tissues, and transversed to various compartments, is under considerable investigation (23). Finally, PSs, once thought to be extremely stable to nucleolytic degradation, are now known to have half lives in the range of less than one hour to greater than 24 hours, in various animal models, depending on the tissue or organ examined. Oligonucleotides with greater stability than PSs will be essential for oral administration of oligomers and will have important implications on improving parenteral administration (24).

Toxicologic Limitations - Dose limiting toxicities for phosphorothioate administration in primates are clotting abnormalities and transient hypertension which likely are related to inhibition of the clotting cascade and activation of the complement pathway, respectively. Toxicities in rodents are quite different, as immune stimulation is most prevalent (25). The polyanionic nature of the oligonucleotides, and particularly phosphorothioates, is often implicated as the cause of these and other toxicity parameters (26-29). The current knowledge of oligonucleotide-protein interactions is sparse and as more is learned, then rationally modifying oligonucleotides will allow modulating protein-oligonucleotide interactions. Research directed to controlling the charge density, lipophilicity and sulfur content (replacement of the thiophosphate linkage) of oligomers is another important medicinal chemistry problem. Dose limiting toxicity in human clinical trials has not been reported.

OLIGONUCLEOTIDE MODIFICATIONS

A diverse range of modifications, at all possible modification sites of an oligonucleotide (Figure 1), have been reported. This chemical data base is quite valuable in continuing medicinal chemistry efforts towards enhancing the drug properties of phosphorothioates. Figure 1 represents a dimer of an oligonucleotide and depicts all the available subunits that may be modified. These subunits are composed of heterocycles, carbohydrates, linkages (backbones), and several types of

connection sites including conjugation sites, as well as complete removal of the sugar-phosphate backbone. Most of the positions available in a G-C or A-T dimer, that do not directly interfere with Watson-Crick base pair hydrogen bonding, have been modified. The antisense concept has well-defined structural requirements for the oligonucleotide ligand that binds to a reasonably characterized receptor (RNA). Although this knowledge is highly valuable, it also provides limits to the scope of potential chemical modifications. A comprehensive structural modification data base, as depicted by Figure 1, is available to search to enhanced drug properties of PSs.

2'- CARBOHYDRATE-MODIFIED OLIGONUCLEOTIDES

Basis for Design - The primary purpose for modifying the carbohydrate subunit (Figure 1), has been to enhance the binding affinity to target RNA and secondarily to enhance nuclease resistance. The principle driving the carbohydrate modifications is to "pre-organize" the antisense oligomer into an "A" form geometry by converting the ribofuranosyl moieties into a 3'-endo conformation (Figure 2) and conforming to torsion angles mimicking dsRNA (30).

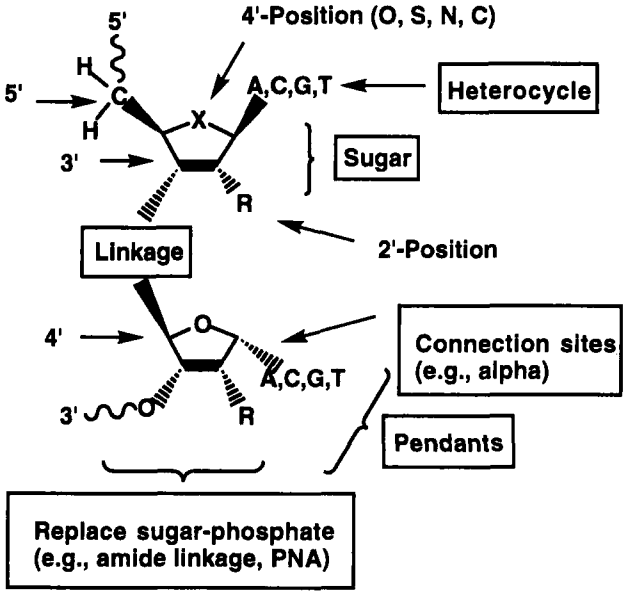

Figure 1. Oligonucleotide modifications

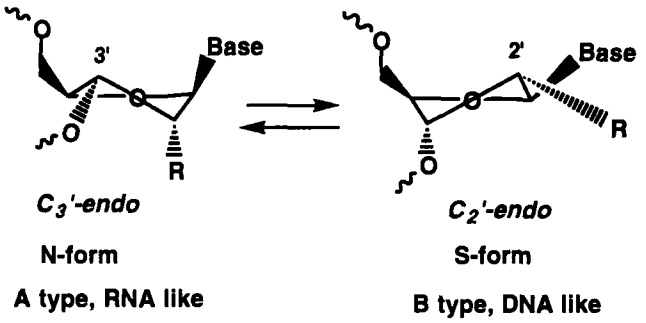

Figure 2. Conformational equilibrium of furanose sugar in nucleic acids

Successful modifications would provide a modified oligomer-RNA duplex resembling the structure of a RNA-RNA duplex which is generally more stable than DNA-RNA or DNA-DNA duplexes (31).

2'-Modified-Oligonucleotides-Structure Property/Activity Relationship Studies - Of the available positions on the β-D-ribofuranosyl moiety to modify, the 2'-position (Figure 1) has proven to be the most valuable, in that a number of modifications have been identified, which markedly enhance several key antisense properties. Placement of a alkoxy or halogen in the 2'-position of a nucleoside has the general effect of shifting the equilibrium between the 2'-endo and 3'-endo to a more 3'-endo conformation. This type of modified oligonucleotide has been referred to as RNA mimics (32). In addition, certain 2'-*O*-modified oligonucleotides possess a high level of nuclease resistance. The identification of 2'-modified oligomers exhibiting enhanced antisense drug properties has resulted from rather extensive structure-property/activity relationship studies performed in the past nine years (1,33). The combination of T_m, melting temperature with the exact complement RNA, and $t_{1/2}$ (half-life) values derived from these studies have set rather high standards for future modification research.

Binding Properties of 2'-*O*-Modified Oligonucleotides - On comparing a series of 2'-*O*-alkyls, it was found that an inverse relationship exists relating the size of the alkyl chain to the binding affinity and the nuclease resistance; the smaller the alkyl group the greater the T_m, whereas nuclease resistance was increased as the alkyl group size increased (33-35). The 2'-fluoro modified oligonucleotides exhibited the greatest increase in binding affinity (ΔT° C /mod +2.3 PS, +2.7 PO). This correlated with the highest 3'-endo sugar conformation reported (36). A study of the direct attachment of a carbon to the 2'-position, that is, 2'-carbon-carbon modified oligonucleotides, was very destabilizing, confirming the need for an electronegative atom in this position (37). In another series of modifications (Figure 3), the 2'-*O*-alkyl chain was substituted with nitrogen and oxygen heteroatoms. 2'-*O*-(aminopropyls), AP, was shown to provide binding affinities about the level of POs (ΔT, 0.0° C PO, +0.7 PS). The most interesting modifications in this series were the oxygen modified 2'-*O*-alkyls, particular methylated ethylene glycols. The 2'-*O*-(methoxyethyl) provided an increase of +2.3° C/mod, PO (38,39).

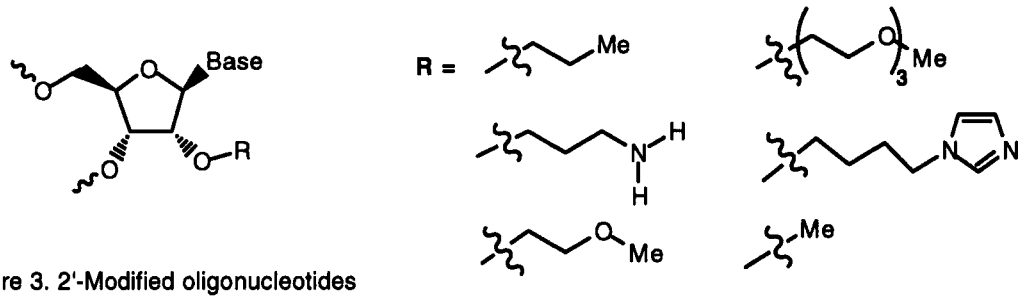

Figure 3. 2'-Modified oligonucleotides

The 2′-*O*-(methoxyethyl), besides providing a 3′-endo conformation, has an additional factor in that the oxygen atom of the ethylene glycol provides a gauche effect with the 2′-oxygen which allows favorable positioning of the group within the minor groove of the heteroduplex. This has been proposed to be the reason for the methoxyethyl-modified oligonucleotides having stronger binding affinities than closely related analogs such as methyl, propyl and methoxypropyl (38-40). Even much longer ethylene glycols, such as 2′-*O*-(methoxytriethoxy), demonstrate high binding affinities (1.8° C/mod) (33).

Stability of 2′-*O*-Modified Oligonucleotides - As more is learned about antisense oligonucleotides, it appears that stability of the oligomer is emerging as a very crucial property. Focusing modification efforts in this area while maintaining a reasonable level of binding affinity may be more important than continuing to design and modify for higher binding affinity of PSs. It is well established that the sulfur in the backbone of PS DNA, as a thiophosphate linkage, primarily contributes nuclease resistance to the oligonucleotide. Whether this level of stability is sufficient for all drug applications of oligonucleotides has rarely been questioned. However, as more data is accumulated, it appears that the level of nuclease resistance of PSs is not as high as originally thought and that greater levels would be beneficial. Furthermore, most of the limitations of PSs, as discussed above, may be correlated to the thiophosphate linkage. Thus, the complete removal of the sulfur or reduction of the level of sulfur in antisense oligonucleotides while increasing the level of nuclease resistance is an important medicinal chemistry objective. 2′-*O*-Modified oligonucleotides that provide greater nuclease resistant than the PS parent yet provide useful binding affinities, may be a solution to this problem. Since PSs are destabilizing (ΔT_m/sulfur is ~ -0.7° C) and 2′-*O*-modifications significantly stabilize (compared to PSs), replacement of PS linkages with PO linkages and adding certain 2′-*O*-modifications should provide favorable binding affinities. The important issue is whether 2′-*O*-modifications can provide nuclease resistance such that phosphodiester linkage can be employed in place of PS linkages.

Enhanced resistance to degradation by nucleases is exhibited by certain 2′-*O*-modifications. For the simple alkyl groups, resistance is correlated to the length the alkyl chain (35). Thus, the greater the bulk emanating from the 2′-position, the greater the $t_{1/2}$ in nuclease assays. The relative stabilities of several 2′-*O*-alkyl-modified RNA have the following order compared to parent PS: OMe < OPr < OPentyl ~ PS. 2′-*O*-Methyl modified PO oligonucleotides are generally about 10-fold less resistant than parent PSs (35). Nuclease resistance was not increased over parent DNA with uniformly modified 2′-fluoro oligonucleotides. Certain substituted 2′-*O*-alkyl-modified PO oligonucleotides provide much greater nuclease resistance. The 2′-*O*-methoxyethyl modification, along with its high binding affinity, exhibits nuclease resistance at approximately the same level as a PS linkage. Further modifications of

the alkyl chain has led to 2′-*O*-alkyl moieties bearing an amine or an imidazole in the chain which is protonated at physiological conditions. These modified oligomers exhibit greatly increased nuclease resistance compared to PSs (35,41). 2′-*O*-(Imidazolybutyl) and 2′-*O*-(aminopropyl)- modified oligonucleotides have shown $t_{1/2}$ with snake venom phosphodiesterase (SVPD) 4-fold and 14-fold greater, respectively, than the PS parent.

A comparison of several 2′-modifications in an uniform or capping motif of a PO or PS 19-mer is depicted in Table 2 (1). Several points should be noted. Only the 2′-*O*-(methoxyethyl),-2′-*O*-(imidazolylbutyl) and 2′-*O* (aminopropyl)-modified oligomers have sufficient nuclease resistance to allow replacement of the PS linkage with the natural PO linkage. Obviously, the same modifications in a PS backbone are extremely stable. The 2′-*O*-methyl modifications will not support use of the natural phosphodiester (PO) backbone. The 2′-*O*-modifications bearing a positive charge (aminopropoxy and imidazolylbutoxy) exhibit remarkable stability towards SVPD.

Modes of Action of 2′-*O*-Modified Oligonucleotides - Chimeric 2′-*O*-Modified Oligonucleotides (GAPmer technology), RNase H-Dependent Mode of Action - Having developed high binding, nuclease resistance 2′-*O*-modified oligonucleotides, it was rather disappointing that oligomers uniformly modified were inactive or less active than their first generation parent PSs. It is now well known that uniformly 2′-*O*-modified-oligonucleotides do not support a RNase H mechanism. The 2′-*O*-modified oligonucleotide-RNA heteroduplex has been shown to present a structural conformation that is recognized by the enzyme but cleavage is not supported (42,43). The lack of activity of 2′-*O*-modified oligonucleotides has led to the development of a chimeric strategy (gapmer technology) (44-46).

This approach focuses on the design of high binding, nuclease resistant antisense oligonucleotides that are "gapped" with a contiguous sequence of 2′-deoxy PSs (Figure 4). On hybridization to target RNA, a heteroduplex is presented that supports a RNase H-mediated cleavage of the RNA strand. The stretch of the modified oligonucleotide-RNA heteroduplex that is recognized by RNase H may be placed anywhere within the modified oligonucleotide. The modifications in the flanking regions of the gap must not only provide nuclease resistance to exo- and endonucleases, but also not compromise binding affinity and base-pair specificity (47).

Table 2. Half-life in minutes of modified oligonuculeotides exposed to SVPD @5X10-3 units/ml

Capped TTT TTT TTT TTT TTX XXX T - 3′

Fully Modified XXX XXX XXX XXX XXX XXX T - 3′

Backbone →	Diester P=O		Thioate P=S	
	Capped	Full	Capped	Full
X = T, 2′-Deoxy		4		240
X = U, 2′-*O*-Methyl	10	15	450	>1440
X = U, 2′-*O*-Propyl	7	80	>1440	>>1440
X = U, 2′-*O*-Pentyl	7		720	

X = U, 2′-Fluoro	3			
X = U, 2′-*O*-(Methoxyethyl)	110			
X = U, 2′-*O*-(Aminopropyl)	1440			
X = U, 2′-*O*-(Imidazolylbutyl)	420			

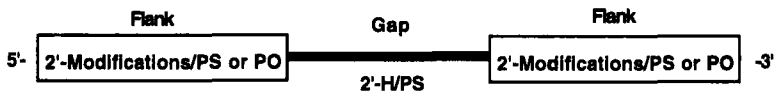

Figure 4. Gapmer technology

Modifications of the phosphorus of the natural phosphodiester linkage to provide methyl phosphonates, phosphorothioates, and phosphoramidates destabilize heteroduplexes -0.7 to -1.5° C for each modification (48-50). The decreased binding affinity of these modified oligonucleotides could be expected to reduce antisense effectiveness. In the case of chimeric 2′-*O*-methoxy or 2′-fluoro modified oligonucleotides, an enhancement in the binding affinity of about 2.0 to 2.3° C (compared to PS) for each modification is obtained (50-52). However, it is now clear that 2′-OMe and 2′-F modified DNA are not sufficiently nuclease resistant to have antisense value as POs backbones (Table 2, 53-56). The potential problem in this area can be circumvented by the use of 2′-OMe or 2′-F modified phosphorothioates in the flanking regions (doubly modified) (51,52).

More recent research has focused on 2′-*O*-modifications, such as methoxyethyl (38) and aminopropyl (41), which not only provide relatively high binding affinities but also a level of nuclease resistance that allows the replacement of the thiophosphate with the natural phosphodiester linkage. 2′-*O*-modifications, with a favorable combination of T_m and $t_{1/2}$, can be employed in the chimera strategy (Gap Technology, Figure 4) which will allow a significant portion of the phosphorothioate linkages to be replaced with PO linkages (Table 2). Just how many sulfurs can be replaced depends on the length of the oligomer and the gap size or RNase H cleavage site. Typically, a 21-mer with a 7-nucleotide gap has 65% of the PS linkages replaced with PO linkages. As noted in the limitations of PSs, reduction of the sulfur content in a PS oligonucleotide could have important implications in the pharmacokinetic and pharmacodynamic properties as well as the toxicity profile of oligonucleotides.

A very important aspect to the gapmer technology is that the gap or RNase H cleavage site must be protected from endonucleolytic cleavage. Phosphodiester linkages and even an alternating PS/PO motif does not provide an useful level of nuclease resistance for biological activity (47,48). A recent report of lack of activity of a "gapped" 3′-amidate is likely due to endonuclease degradation (57). Uniform phosphorothioates are the only useful modification to allow a reasonable combination of binding affinity and nuclease resistance and also support an RNase H mechanism.

An informative depiction of the concept of a combination of modifications and the balance or optimization between binding affinity and nuclease resistance using a gapmer strategy has been presented (Figure 5) (1). The sequence examined is ISIS-5132, a 20-mer and as an uniform 2′-deoxy/PS is in Phase II antitumor clinical

trials. The present biophysical study examined the binding affinity (T_m) and the nuclease resistance ($t_{1/2}$) of a modified gapmer, that is, the flanks were POs rather than the usual PSs. The binding affinity of ISIS-5132 (full 2′-deoxy/PS) is about 62° C, the lowest of the oligomers in the study, but its $t_{1/2}$ is very high because of the PS linkages. In comparison, the ISIS-5132 sequence, as a full 2′-deoxy/PO, has higher binding affinity than the 5132/PS, as expected because of the removal of the destabilizing sulfur in the backbone, but this modified oligomer is rapidly degraded by SVPD ($t_{1/2}$ ~ 5 mins). The 2′-fluoro modification in the flanks provides the highest binding affinity (2.3° C /mod) but the lowest nuclease resistance. As can be seen from Figure 5, the best combination of binding affinity and nuclease resistance is exhibited by the 2′-*O*-(methoxyethyl) modification, where the nuclease resistance is the highest of the oligomers studied and the binding affinity is almost as high as the 2′-fluoro oligomer. It is interesting to note that the 2′-*O*-methyl modification is not very stable as a modified gapmer and is unlikely to allow removal of the sulfur from antisense oligonucleotides. Hence the 2′-*O* methyl modification requires an PS backbone. The 2′-*O*-propyl also has a favorable combination of properties. *In vitro* and *in vivo* antisense studies of the oligomers in this study were nicely correlated with the observed biophysical properties. It is also important to note that in this modified gapmer 53% of the sulfurs (10 of the 19 phosphorothioates) were converted to the natural phosphodiesters linkages.

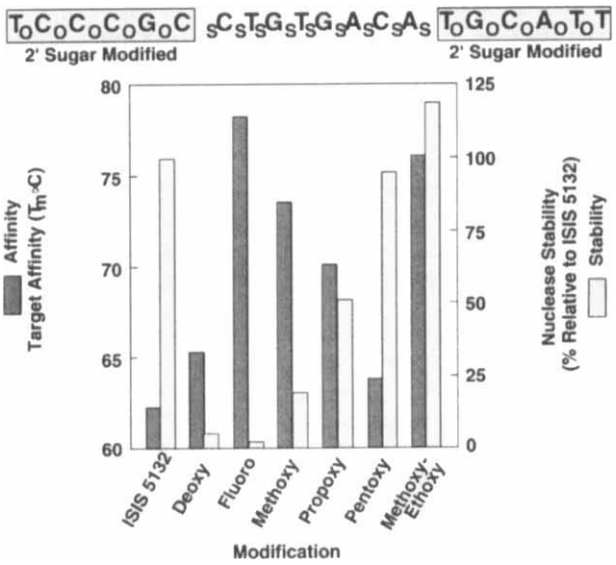

Figure 5. Biophysical properties of 2′-sugar-modified chimeric oligonucleotide derivatives of ISIS 5132. The sequence of oligonucleotide ISIS 5132 is shown at the top with nucleotides that contain 2′-sugar modifications indicated: phosphodiester linkages are indicated with a subscript 'o' and phosphorothioate linkages are indicated with a subscript 's'. Melting temperatures (target affinity) were determined as described (33). Relative nuclease stability was determined against snake venom phosphodiesterase as described previously (37). Quanitiation was also as described previously (58).

2′-*O*-Modified Oligonucleotides with a RNase H-Independent Mode of Action - Another potential mode of action for the high binding, nuclease resistant 2′-*O*-modified

oligonucleotides is direct, sequence specific binding to a site on the targeted RNA sequence. RNase H degradation of the targeted RNA is not involved in this case as uniformly 2′-*O*-modified oligonucleotides-RNA heteroduplexes, although binding to RNase H, are not cleavaed by the enzyme (59).

The first report of a RNase H-independent antisense oligonucleotide having greater activity than its parent PSs (which supports RNase H), describes targeting of the 5′-cap region of human ICAM-1 transcript in HUVEC cells with a series of uniformly 2′-*O*-modified 20-mer oligonucleotides (60). These RNase H-independent oligomers did not affect splicing or transport of the ICAM-1 mRNA, but instead selectively inhibited formation of the 80S translation initiation complex. The 2′-*O*-(methoxyethyl)/PO oligomer demonstrated the greatest activity with a IC_{50} of 2.1 nM (T_m 87.1° C) and its PS analog had an IC_{50} of 6.5 nM (T_m 79.2° C). Correlation of activity with binding affinity was not always followed as the 2′-F/PS (T_m 87.9° C) was less active than the 2′-*O*-(methoxyethyl) PS (T_m 79.2° C) by 4-fold. The 2′-*O*-methoxy/PS analog had an IC_{50} of >50 nM (T_m 76.5° C). The RNase H competent 2′-deoxy/PS parent oligonucleotide (T_m 76.4° C) exhibited an IC_{50} of 41 nM.

Pharmacokinetic Properties of 2′-Modified Oligonucleotides - Several reports of *in vivo* pharmacokinetics of 2′-*O*-modified oligonucleotides in mice have been described (61,62). Doubly modifying a sequence with a PS backbone and 2′-*O*-propyls at the 3′-end significantly increased the stability towards 3′-exonucleases in the tissues examined. A modified gapmer and a uniformly modified 2′-*O*-propyl/PO, both having the 2′-*O*-propyl as a single modification at the 3′-end, were degraded in plasma at about the rate of parent PS.

The pharmacokinetic behavior of the C-*raf* sequence ISIS 5132 described above, as the parent PS and the modified gapmer (8 PS in the gap, 11 2′-O-methoxyethyls/PO flanks) was examined in tumored nude mice (38). The gapmer was cleared much more rapidly as indicated by higher concentrations in the kidney and urine than the parent PS. Other organs demonstrated similar concentrations, especially at the early time points. The antitumor activity of the gapmer was substantially increased over the parent PS.

A series of 5′-fluorescein labeled poly A PSs 10-mers bearing various 2′-*O*-modifications (nonyl, pentyl, propyl, methyl, fluoro and hydrogen (deoxy) with different lipophilicities were examined for their ability to transverse synthetic liposomal membranes (63,64). The 2′-*O*-propyl and 2′-fluoro analogs exhibited significantly shorter effux half-lives across the membranes than the other modified oligomers. The greater lipophilic nonyl and pentyl modified oligomers when encapsulated with [^{14}C] sucrose, demonstrated significant shorter efflux half-lives compared to the other analogs. The results of this study suggest that it is possible to modify the membrane permeation characteristics of oligonucleotides by means of 2′-modifications.

Toxicity Implication of 2′-Modified Oligonucleotides - The phosphorothioate linkage has been suggested, in very early antisense research, to bind more tightly to proteins (22,26) and, therefore, likely to cause toxicities. A C-*raf* sequence modified with 2′-*O*-methoxyethyl)/PO sequence in the 3′ and 5′-ends and an internal gap of eight PS/2′-deoxys (5′-*To*Co*Co*Co*Go*Co--CsTsGsTsGsAsCsAs--*To*Go*Co*Ao*T*sT) was examined in an assay to determine clotting time vs. concentration (APTT assay). This sequence, having 10 PS linkages replaced with POs because of sufficient nuclease

resistance provided by the 2′-*O*-methoxyethyls, exhibited a significantly reduced clotting time compared to the parent PS having 19 PS linkages. The concentration of the parent PS and the modified gapmer required to double the clotting time were 12.1 and > 53 mM, respectively (33).

Summary of 2′-Sugar-Modified Oligonucleotides - In summary, the 2′-*O*-modifications are relatively easy to synthesize and will be less expensive than their deoxy counterparts. They offer high binding affinities due to a greater 3′-endo conformation and certain modifications, such as aminopropyl and methoxyethyl, provide superior nuclease resistance compared to PSs. Unlike other subunits to modify, a 2′-modification motif has available many sites to modify to alter various antisense properties. Thus, at this stage of the process of making drugs out of oligonucleotides, the 2′-modification area has clearly demonstrated superior properties relative to modifications of other oligonucleotide subunits.

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SECTION VII. TRENDS AND PERSPECTIVES

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Chapter 32. To Market, To Market - 1997

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During 1997, the first time introduction of new chemical entities (NCEs) for human therapeutic use into the world market totaled 39 (1). This number of NCEs is a slight increase from 38 in 1996 (2), an increase from 36 in 1995 (3), 1992 (4) and 1991 (5) and a decrease from 44 in 1994 (6) and 43 in 1993 (7).

The US, as in 1996, took the number one position for new introductions with 12 NCEs, down from last years 17. In a repeat of last year, the UK was second with 8 NCEs followed by Germany in third with 6 NCEs. Japan was fourth with 4, while France and Italy both tied for fifth position with 2 NCEs each. Denmark, the Netherlands, Spain, Sweden and Switzerland all were represented with 1 NCE. A similar trend is observed when one examines the originators of NCEs. The US ranked first with 10, followed by Japan and the UK with 7 and 6 NCEs, respectively. Germany was next with 4. France and Switzerland tied with 3 each. Italy ranked next with 2 NCEs. Israel, Norway, Spain and Sweden all had 1 NCE each.

The NCEs launched in 1997 came to market with the fastest approval time to date. The median time was 13.4 months compared with 14.3 months in 1996. Drugs given priority status received an accelerated review because it was recognized that they would provide a major advancement in medical treatment. The following four drugs were given this status: Agrylin, Rescriptor, Rezulin and Viracept. Agrylin was the first therapy for elevated platelet count with essential thrombocytopenia. The second generation nonnucleoside reverse transcriptase inhibitor Rescriptor for HIV-1 infection is a member of the bis(heteroaryl)piperazine class. Rezulin was the first drug approved that works at the cellular level to improve insulin resistance. Viracept was the first HIV protease inhibitor approved for adult and pediatric formulations. There were other compounds that were first in class or had novel modes of action but received standard priority. Copaxone is an immunomodulator that was the first alternative to INF-β for multiple sclerosis. Posicor is the first in a new class of calcium channel blockers, while Tasmar is the first example of a catechol-O-methyltransferase (COMT) inhibitor for use in Parkinson's disease. Zorac is a new generation of topical, receptor selective retinoids. The year was also notable for the number of drugs launched that were not first in class. Aricept and Exelon are two new drugs for treatment of Alzheimer's disease that are both cholinesterase inhibitors and are similar in action to Tacrine the first anticholinesterase for Alzheimer's disease. Imigran was the first product for the treatment of migraines but Zomig and Naramig are two new drugs for this indication. Zyflo, a 5-lipoxygenase inhibitor, was launched for asthma

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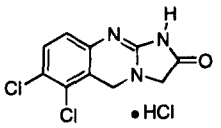
and gives Accolate competition. Lipitor achieved phenominal market share in the statin arena which may be considered a fairly mature market.

Anagrelide Hydrochloride (hematological) (8-13)

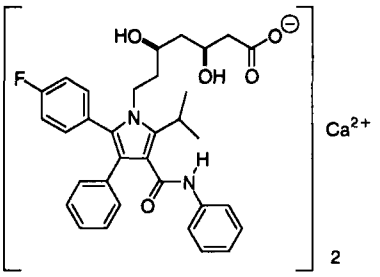
Country of Origin:	US
Originator:	Roberts
First Introduction:	US
Introduced by:	Roberts
Trade Name:	Agrylin
CAS Registry No.:	58579-51-4
Molecular Weight:	292.55



Agrylin was launched in the US for thrombocytosis (essential or associated with chronic myelogenous leukemia). The imidazoquinazoline derivative can be prepared from 2,5,6-trichloro-3,4-dihydropyrimidine via alkylation with ethyl bromoacetate followed by heating with ethanolic ammonia or treatment of N-(2-amino-5,6-dichlorobenzyl)glycine ethyl ester with cyanogen bromide. As a result of its anti-cAMP phosphodiesterase (PDE III) activity, it was initially tested as a platelet aggregation inhibitor. However it was found that at much lower concentrations it became thrombocytopenic. While the mechanism is not fully understood, it did not shorten platelet survival nor inhibit the formation of colony-forming units-megakaryocytes (CFU-M) but primarily interfered with the maturation of megakaryocytes (reduction in size with altered ploidy). It did decrease peripheral vascular resistance and had a positive inotropic effect.

Atorvastatin Calcium (dyslipidemia) (14-20)

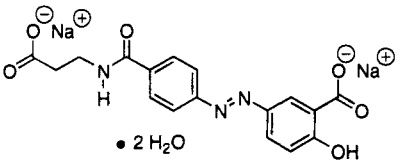
Country of Origin:	US
Originator:	Parke-Davis
First Introduction:	UK
Introduced by:	Parke-Davis
Trade Name:	Lipitor
CAS Registry No.:	134523-03-8
Molecular Weight:	1155.37



Lipitor was launched in Canada, the Netherlands, the UK and the US as an orally-active hypocholesterolemic agent. It was the first pharmaceutical product ever to attain over one billion dollars in sales in its first year. It can be synthesized by a number of routes but the most efficient involves the Paal-Knorr reaction of an acetonide protected dihydroxy amino ester and a diaryl phenylacetamide diketone. Lipitor is a liver selective, reversible competitive inhibitor of HMG-CoA reductase, the rate limiting step in cholesterol biosynthesis. Lipitor monotherapy resulted in a reduction of LDL cholesterol by up to 60%. Lipitor is about 2-4 times more potent, on a dosage basis, than Simvastatin. The superior properties of Lipitor can be attributed to its greater uptake and longer duration of action in the liver. In addition to its effects on cholesterol, Lipitor is also effective in lowering triglycerides. While the mechanism is not clear, two theories proposed are: a) the decrease in cholesterol causes a concomitant increase in hepatic LDL-receptor activity (mostly B and E type) which results in a decrease in triglycerides through an increase in binding of triglycerides to VLDL and LDL, and b) the decreased level of cholesterol impairs VLDL transport of triglycerides.

Balsalazide Disodium (ulcerative colitis) (21-27)

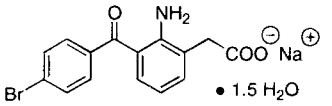
Country of Origin:	UK
Originator:	Biorex
First Introduction:	UK
Introduced by:	Astra
Trade Name:	Colazide
CAS Registry No.:	80573-04-2
Molecular Weight:	437.32



Colazide was launched in the UK for mild to moderate acute attacks of ulcerative colitis. It can be prepared by diazotization of 3-(4-aminobenzoyl-amino)propionic acid followed by condensation with salicylic acid. Colazide, an analogue of sulfasalazine, was found to be non-toxic and non-mutagenic in the Ames test. Once ingested the molecule releases a non-toxic fragment, 4-aminobenzoyl-β-alanine (poorly absorbed - 75% excretion in stool), and the active component 5-aminosalicylic acid (Mesalazine). The exact mechanism of action is not clearly understood but it is cytoprotective and has antiinflammatory properties. Other biological effects have been observed and include the following: (a) granulocyte activation is blocked, (b) there is a reduction in myeloperoxidase activity, and (c) a reduction in the release of arachidonic acid with a concomitant decrease in prostaglandin and leucotriene (as indicated by the lack of production of LTB_4) production. It can behave as a reactive oxygen species (ROS) scavenger, inhibit PAF formation, reduce IL-1 production, and antagonize TNF and NK cells. While comparable in efficacy to sulfasalazine, Colazide removes the possibility of sulfasalazine side-effects, such as, agranulocytosis, hepatotoxicity and male infertility.

Bromfenac Sodium (NSAID) (28-30)

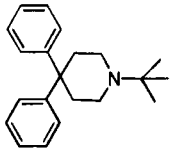
Country of Origin:	US
Originator:	American Home Products
First Introduction:	US
Introduced by:	Senju
Trade Name:	Duract
CAS Registry No.:	120638-55-3
Molecular Weight:	383.18



Duract was launched in the US as a potent, orally-active, long lasting peripheral analgesic with antiinflammatory properties. It is structurally similar to ketoprofen and diclofenac and can be prepared in three steps from 2-amino-4'-bromophenone using Gassman's oxindole synthesis. Duract's biological effects are a result of its ability to reduce prostaglandin production through inhibition of cyclooxygenase. As a 4-bromo derivative of Amfenac, this modification increased the duration of analgesic activity and antiinflammatory potency. It was also free of any CNS, cardiovascular or autonomic effects. In comparison, 5 mg of Duract was equipotent to 650 mg of ASA and 25 mg was slightly more potent than 400 mg of Ibuprofen.

Budipine (anti-Parkinsonian) (31-35)

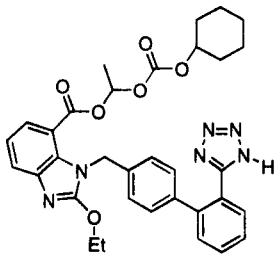
Country of Origin:	Germany
Originator:	Byk Gulden
First Introduction:	Germany
Introduced by:	Promonta Lundbeck
Trade Name:	Parkinsan
CAS Registry No.:	57982-78-2
Molecular Weight:	293.45



Parkinsan was launched in Germany as a centrally-active anti-Parkinsonian agent. It can be prepared in three steps: a Mannich-aldol sequence to assemble the 4-phenylpiperidine scaffold followed by a Friedel-Crafts reaction to introduce the second phenyl ring. Parkinsan is effective for the treatment of Parkinsonian tremors and is similar in action to, but more potent than, biperiden. It exhibits use-dependent, open channel, uncompetitive NMDA receptor antagonistic activity. This may occur by binding to the PCP site in addition to interacting with sigma, binding sites in the frontal cortex. Parkinsan is also an antagonist at presynaptic muscarinic autoreceptors but facilitation of direct or indirect dopaminergic transmission does not contribute to its actions. While its mechanism of action is not completely understood, it has a weak inhibitory effect on dopamine reuptake, inhibits evoked GABA release (with low affinity for GABA-A receptors and a lower affinity for benzodiazepine receptors), and has a weak inhibitory effect on MAO-B.

Candesartan Cilexetil (antihypertension) (36-41)

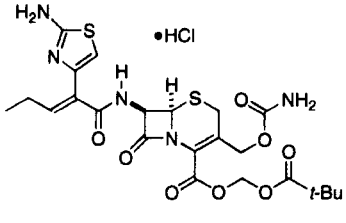
Country of Origin:	Japan
Originator:	Takeda
First Introduction:	Sweden
Introduced by:	Astra
Trade Name:	Atacand
CAS Registry No.:	145040-37-5
Molecular Weight:	440.46



Atacand was launched in Australia, Belgium, Canada, Denmark, Finland, the Netherlands, Norway, Sweden, S. Africa and the US as an antihypertensive agent. It can be prepared from 3-nitrophthalic acid in about eight steps. Pharmacological studies have indicated Atacand is about 10-fold more potent than Losartan and has a long elimination half-life. Like losartan, Atacand is converted to its active form during GI absorption (via ester hydrolysis). It is a potent antagonist of angiotensin II type 1 receptors. This occurs through tight binding and slow dissociation, and is more potent than ACE inhibitors. It is well tolerated (can be taken by elderly and those with type II diabetes) and has no gender effects.

Cefcapene Pivoxil (antibiotic) (42-47)

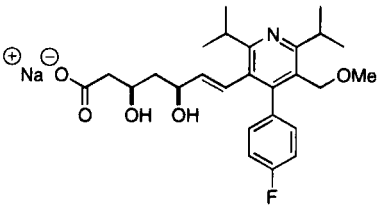
Country of Origin:	Japan
Originator:	Shionogi
First Introduction:	Japan
Introduced by:	Shionogi
Trade Name:	Flomox
CAS Registry No.:	105889-45-0
Molecular Weight:	604.09



Flomox was launched in Japan as an orally active cephalosporin for respiratory and urinary tract infections, heptatic infections, ophthalmological and otorhinolarynological infections, skin/soft tissue infections, and for use in gynacology, dentistry and oral surgery. It can be prepared by condesation of 2(Z)-(2-(*t*-butoxycarbonylamino)thiazol-4-yl)-2-pentenoic acid with 7-amino-3-(carbanoyl-oxymethyl)-3-cephem-4-carboxylic acid pivaloyl methyl ester followed by deprotection. Flomox is highly active against a wide variety of Gram-positive and Gram-negative bacteria, except for several strains such as *Pseudomonas aeruginosa* and enterococci, by acting as a cell wall synthesis inhibitor (β-lactamase stability against TEM-1 type β-lactamases) and is more effective than cefaclor and cefdinir. Absorption is improved by the pivaloyloxymethyl ester group which is easily lost by deesterification during GI absorption to produce the biologically active form. The pivalic acid generated quickly conjugates with carnitine and is excreted in the urine. The drop in plasma levels of carnitine was dose dependent and returned to normal levels upon termination of treatment.

Cerivastatin (dyslipidemia) (48-52)

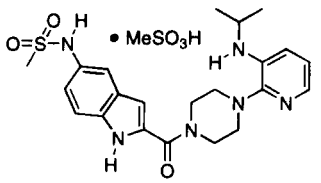
Country of Origin:	Germany
Originator:	Bayer
First Introduction:	UK
Introduced by:	Bayer
Trade Name:	Lipobay
CAS Registry No.:	143201-11-0
Molecular Weight:	481.54



Lipobay was launched in Denmark, Germany and the UK for treatment of primary hypercholesteremia types IIa and IIb. It is available through a nine step synthesis beginning with the Hantsch reaction of ethyl 3-amino-4-methylpent-2-enoate and 4-(ethoxycarbonyl)-5-(4-fluorophenyl)-2-methylpent-4-en-3-one. Lipobay acts as an HMG-CoA reductase inhibitor showing a high liver selectivity (50 fold). *In vitro* Lipobay had a higher affinity (110x) for the enzyme than does Lovastatin, Simvastatin and pravastatin which resulted in a lower ED_{50} *in vivo* compared to Lovastatin. Three metabolites generated by the CYP3A4 enzyme system were equal to the parent in activity. Lipobay was as effective as Gemfibrozil in type IIb hypercholesteremia and had no age or gender effects. It decreased plasma levels of VLDL and LDL cholesterol by a mechanism similar to other HMG-CoA reductase inhibitors. The (-)-antipode is completely inactive indicating a high stereospecificity by the enzyme. It is therapeutically useful at ultra-low doses which may minimize any drug-drug interactions.

Delavirdine Mesylate (antiviral) (53-59)

Country of Origin:	US
Originator:	Pharmacia & Upjohn
First Introduction:	US
Introduced by:	Pharmacia & Upjohn
Trade Name:	Rescriptor
CAS Registry No.:	147221-93-0
Molecular Weight:	552.68

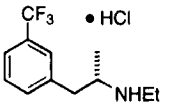


Rescriptor was launched in the US for HIV positive individuals. It can be prepared in seven steps from 2-chloro-3-nitropyridine and piperazine. Rescriptor is a

member of the bis(heteroaryl)piperazine (BHAP) class of non-nucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs) of which it is a second generation drug. It acts exclusively as an allosteric mixed inhibitor of both RNA- and DNA-directed polymerase domains of RT and has a much higher binding affintiy for the enzyme-substrate complex than for the free enzyme. Rescriptor does not inhibit RNase H activity of RT. It prevented the spread of HIV significantly longer than AZT. Development of resistance to Rescriptor can occur, however the mutated strain becomes more vulnerable to other members of the NNRTIs. It is metabolized in the liver by CYP3A.

Dexfenfluramine (antiobesity) (60-66)

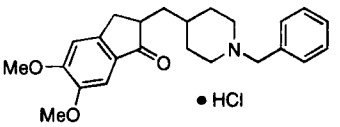
Country of Origin:	France
Originator:	Servier
First Introduction:	France
Introduced by:	Servier
Trade Name:	Redux
CAS Registry No.:	3239-45-0
Molecular Weight:	267.72



Redux, having been already launched in Europe, was marketed in Canada and the US for obesity. The (+)-isomer of fenfluramine is obtained by resolution using d-camphoric acid and ultimate conversion to the HCl salt. The levo-isomer has significant effects on dopaminerigic neurotransmission, while the dextro-isomer has a greater anorectic effect because it is more selective on serotonin as a 5-HT agonist with no dopaminergic or noradenergic activity. Redux inhibits the presynaptic release of serotonin, is a reuptake inhibitor, a 5-HT_{1B} receptor agonist, and a postsynaptic 5-HT_{2C} receptor agonist . A dietary reduction in fat intake was thought to proceed via a CCK A-type receptor agonist activity. Due to the side effects of primary pulmonary hypertension, brain serotonin neurotoxicity and valvular heart disease, Redux was withdrawn world wide.

Donepezil Hydrochloride (anti-Alzheimer) (67-75)

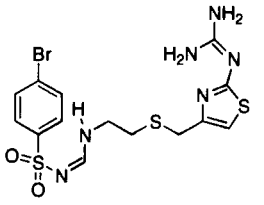
Country of Origin:	Japan
Originator:	Eisai
First Introduction:	US
Introduced by:	Pfizer
Trade Name:	Aricept
CAS Registry No.:	120011-70-3
Molecular Weight:	415.96



Aricept was launched in Canada, Germany, the UK and the US for treatment of mild to moderate Alzheimer's disease and dementia. It was prepared in three steps beginning with the condensation of 5,6-dimethoxy-1-indanone with 1-benzylpiperidine-4-carboxaldehyde. Aricept is a reversible, non-competitive inhibitor of acetylcholinesterase. It is 570-1250 times more selective for acetylcholinesterase than for butylcholinesterase. It has a greater affinity for brain over peripheral acetylcholinesterase with no inhibition in cardiac and smooth muscle, but with slight effects in striated muscle. Aricept inhibited all three isozymes (type A, G2 and G4) of acetylcholinesterase thereby delaying the decline of cholinergics found in the AD brain and slowing the loss of cognitive abilities. It has a 60-70 hr half-life, readily penetrates the CNS, and is 100% bioavailable through the gut with no hepatotoxicity.

Ebrotidine (antiulcer) (76-81)

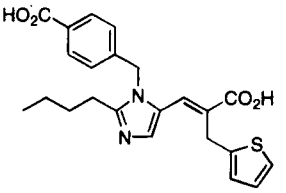
Country of Origin:	Spain
Originator:	Ferrer
First Introduction:	Spain
Introduced by:	Ferrer
Trade Name:	Ebrocit
CAS Registry No.:	100981-43-9
Molecular Weight:	477.43



Ebrocit was launched in Spain as a gastroprotective agent. It was prepared from 4-bromobenzenesulfonamide in two steps. Ebrocit's activity arises from its ability to antagonize histamine H_2-receptors (the first of a new generation) showing 1.5-2.5 times higher affinity than Ranitidine and 2-fold greater affinity than cimetidine which correlates to an antisecretory potency of 1 times and 4-10 times respectively. Cyto- and gastroprotection arises from increased gastric mucus production, enhanced physiochemical properties (decreased permeability of H^+), and an increase in mucin glycosylation and sulfation. It acts on gastric mucosal EGF and PDGF receptor expression. Nitric oxide was also found to be involved. Ebrocit caused enhanced mucosal blood flow with anti *H. pylori* activity. It has diminished P450 binding which eliminates the possibility of mutagenic nitrosoamine formation.

Eprosartan (antihypertensive) (82-89)

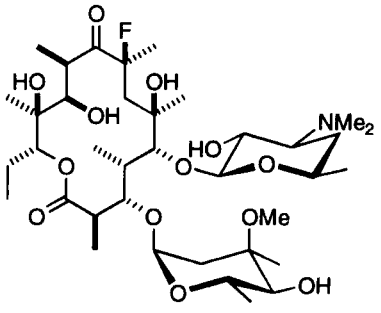
Country of Origin:	UK
Originator:	SmithKline Beecham
First Introduction:	Germany
Introduced by:	SmithKine Beecham
Trade Name:	Teveten
CAS Registry No.:	133040-01-4
Molecular Weight:	424.52



Teveten was launched in Germany for the treatment of hypertension. There are several ways in which it has been prepared, the shortest of which is four steps; beginning with displacement of 2-butyl-4-chloroimidazole-5-carboxaldehyde with methyl 4-(bromomethyl)benzoate. Teveten is an angiotensin II antagonist selective for the AT_1 subtype receptor. It is a potent, highly selective, competitve antagonist with no agonist activity. Duration of action is similar to Enalapril (greater than 12 hr) but Teveten had a faster onset. While it is orally active, it rapidly dissociates from the receptor. This is contrary to its prolonged duration of action, which presumably results from slow removal from compartments within tissue, cells or matrix around the AT_1 receptor. It is not bound by BSA.

Flurithromycin Ethylsuccinate (antibiotic) (90-97)

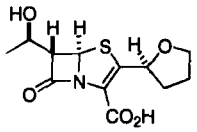
Country of Origin:	UK
Originator:	Pharmacia & Upjohn
First Introduction:	Italy
Introduced by:	Pierrel
Trade Name:	Ritro
CAS Registry No.:	82730-23-2
Molecular Weight:	751.93



Ritro was launched in Italy as an antibacterial agent. It can be prepared by fluorination of 8,9-anhydroerythromycin A 6,9-hemiketal N-oxide followed by reduction of N-oxide. Macrolide antibiotics bind to 50S ribosomal subunits and inhibit protein synthesis. The spectrum of activity is similar to erythromycin (Gram-positive, Gram-negative and anaerobes), however, the fluorine substitution gives it characteristics opposite to that of erythromycin. For example, Ritro has improved acid stability, a prolonged serum half-life (4 hr), higher tissue penetration (10 times extracellular concentration), and better bioavailability. It can be administered with other antibiotics and used in the treatment of serious nosocomial respiratory infections since it has low liver toxicity and no hepatic interactions.

Fropenam (antibiotic) (98-106)

Country of Origin:	Japan
Originator:	Suntory
First Introduction:	Japan
Introduced by:	Suntory
Trade Name:	Farom
CAS Registry No.:	106560-14-9
Molecular Weight:	285.32



Farom was launched in Japan for use as an antibiotic against common respiratory tract pathogens. It can be prepared by several related routes of about seven steps starting with tetrahydrofuran-2-thiocarboxylic acid and a silylated azetidinone. It is a broad spectrum oral penem antibiotic that is β-lactamase stable. Farom is the most active β-lactam against anaerobes (more than cefaclor, cefixime and amoxicillin) but also has activity against Gram-positive, Gram-negative and enterobacteriaceae. It is equally active against strains carrying plasmid and chromosome mediated β-lactamases. The short plasma elimination half-life is the result of hydrolysis by renal dehydropeptidase. It is more stable to hydrolysis by extended spectrum β-lactamases than some second and third generation cephalosporins.

Glatiramer Acetate (Multiple Sclerosis) (107-114)

Country of Origin:	Israel
Originator:	Yeda
First Introduction:	US
Introduced by:	Israel
Trade Name:	Copaxone
CAS Registry No.:	28704-27-0
Molecular Weight:	14-23 kDa

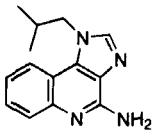
Poly[L-Glu$^{13-15}$, L-Ala$^{39-46}$, L-Tyr$^{8.6-10}$, L-Lys$^{30-37}$]$_n$ AcOH n=15-24

Copaxone was launched in Israel and the US for treatment of relapsing-remitting multiple sclerosis. The amino acid polymer is prepared from the N-carboxyanhydrides of Tyr, Ala, γ-benzylglutamate and ε,N-trifluoroacetyllysine followed by deprotection. Structurally, the random polymer, with a residue molar ratio of 6.0:1.9:4.7:1.0=Ala:Glu:Lys:Tyr, simulates myelin basic protein (MBP). This gives it immunomodulating and immunosuppressive activity (antigen specific so not a general immunosuppressive). The mechanism involves binding to MHC class II (I-A/DR) molecules which results both in competition with myelin antigens for T-cell activation and in induction of specific suppressor cells of the Th2 type. Therefore, the antigen-specific interaction gives rise to a reduced probability of long-term damage to the

immune system. The competition with MBP and other myelin-associated antigens inhibits T-cell responses to MBP, and the binding occurs with high efficiency, fast rates and is non-species specific. It resulted in a 29% reduction in relapse rate and is most effective in patients with less accumulated neurologic disability.

Imiquimod (antiviral) (115-122)

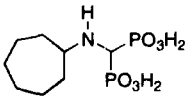
Country of Origin:	US
Originator:	3M Pharmaceuticals
First Introduction:	US
Introduced by:	3M Pharmaceuticals
Trade Name:	Aldara
CAS Registry No.:	99011-02-6
Molecular Weight:	240.31



Aldara was launched in the US for the topical treatment of genital warts caused by human papillomavirus (HPV). It can be prepared in a six step approach beginning with the nucleophilic substitution of 4-chloro-3-nitroquinolone with isobutylamine or via a thermal electrocyclic ring closure of 1- and 2-azahexatriene systems. Aldara has antiviral and antitumor properties, with the former activity arising from the induction of cytokines, in particular, INF-α. Aldara also induces TNF-γ, IL-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, GM-CSF, G-CSF and MID-1α formation 1-4 hr after stimuli. The exact cells responsible for the response have not been determined, however, it is not activating T lymphocytes, NK cells, B lymphocytes, or dendritic stem cells but monocytes ($CD14^+$, $CD36^+$, HLA-DR^+, HLA-$DQ^\pm$, $CD19^-$, $CD16^-$ and $CD23^-$) are partly responsible. The speculation is that Aldara may interact with a kinase modulating the transduction pathway leading to cytokine genes. There is no direct antiviral activity (induction of IFN does not follow true dose response) and it is not mutagenic. The hydroxylated metabolite also induces IFN-α.

Incadronic acid (osteoporosis) (123-130)

Country of Origin:	Japan
Originator:	Yamanouchi
First Introduction:	Japan
Introduced by:	Yamanouchi
Trade Name:	Bisphonal
CAS Registry No.:	124351-85-5
Molecular Weight:	287.19



Bisphonal, a new third generation drug, was launched in Japan for the treatment of osteoporosis. It can be prepared in two steps by heating cycloheptylamine, triethylorthoformate and diethyl phosphite followed by ester hydrolysis. The mechanism of action is not well understood. Bisphonal has no effect on bone mineralization but tightly binds to calcified bone matrix and inhibits bone resorption. It causes a decrease in the number and activity of osteoclasts (morphology and decrease in H^+ secretion). They seem to undergo cell death with an impairment in the process of osteoclast formation. Bisphonal does not induce osteomalacia and its activity is partly mediated through osteoblasts (releases an inhibitor of osteoclast recruitment). It is a potent inhibitor of squalene synthase (50-fold) maybe by Mg^{2+} chelation or as a pyrophosphate mimic, undergoes practically no metabolism, and stimulates renal production of 1,25-dihydroxyvitamin D.

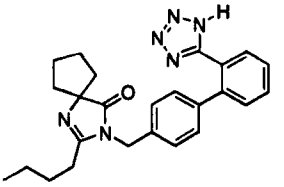
Interferon Alfacon-1 (antiviral) (131-135)

Country of Origin:	US	Trade Name:	Infergen
Originator:	Amgen	CAS Registry No.:	74899-72-2
First Introduction:	US	Molecular Weight:	19,600
Introduced by:	Amgen		

Infergen was launched in the US for the treatment of chronic hepatitis C. The protein was prepared by examining the sequences of naturally occurring IFN-α's (14 different types) and assigning the most frequently observed amino acid in each corresponding position. The synthetic DNA coding this consensus peptide of 166 amino acids was ligated into an E. coli expression vector, produced by bacterial cell fermentation and purified to homogeneity (>98%). Infergen has 30% identity with INF-β and 60% identity with IFN-ω. It has the correct disulfide bridges and was structurally similar to IFN-α. There are three isoforms of IFN's which results in differing antiviral properties, namely, the extent of NK cell activation, the capacity to induce IL-1, and the presentation of class I HLA antigen. Infergen was more effective than the other IFN's with a 10-20 fold greater activity than IFN-α_{2a} and IFN-α_{2b}, had a greater antiproliferative effect and was more effective at activating NK cells. In addition, it had immunomodulatory effects similar to IFN-β_{1b} and IFN-α_{2a}.

Irbesartan (antihypertensive) (136-142)

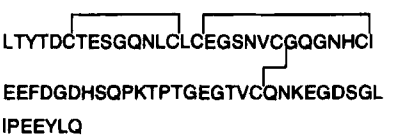
Country of Origin:	France
Originator:	Sanofi
First Introduction:	UK
Introduced by:	Sanofi
Trade Name:	Aprovel/Avapro
CAS Registry No.:	138402-11-6
Molecular Weight:	428.54



Avapro was launched in Germany, the UK and the US for hypertension. It can be prepared in six steps starting with cyclopentanone or in three steps from 1-aminocyclopentanecarboxylic acid ethyl ester and pentanimidic ethyl ester. Avapro is an angiotensin II receptor antagonist that is non-competitive and selective for AT_1 subtypes and has no AT_2 activity at postsynaptic receptors compared to presynatpic. It has no affinity for various non angiotensin II receptor types in binding, no interaction with calcium channels or antiports, and no affinity for α_1- and α_2-adrenoreceptors, serotonergic receptors, muscarinic m_1 and m_2 or other receptors. It is as potent as saralasin but with no agonist activity and is 10 times more potent than DuP753 in rats. It is similar in efficacy to enalapril (in those with severe hypertension) and atenolol, while more effective than losartan for mild to moderate hypertension.

Lepirudin (anticoagulant) (143-152)

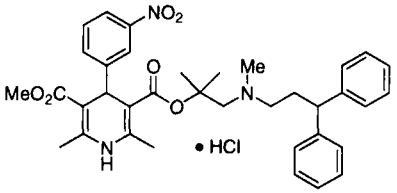
Country of Origin:	Germany
Originator:	Hoechst Merion Roussel
First Introduction:	Germany
Introduced by:	Hoechst Merion Roussel
Trade Name:	Refludan
CAS Registry No.:	8001-27-2
Molecular Weight:	6,979



Refludan was launched in Germany for heparin-associated thrombocytopenia. Originally isolated from the saliva of leeches (*Hirudo medicinalis*), the gene for recombinant hirudin was synthesized and expressed in *Saccharomyces cerevisiae*. The isolated protein is 65 amino acids differing from the native protein by the first two amino acids (Leu1 and Thr2) and Tyr63 is not sulfated, while it does retain the three disulfide bonds. Refludan is a potent, specific and almost irreversible inhibitor of thrombin with which it forms a 1:1 complex. The N-terminal domain binds to the active catalytic site of thrombin, while the C-terminal end binds to the anion binding exosite (fibrinogen binding cleft). It has several advantages over heparin: it can inhibit thrombin bound to extracellular matrices, does not require antithrombin III as a cofactor, is not inhibited by activated platelet nor increases platelet activity, and is a pure, single compound with a single mechanism of action. It is useful for myocardial infarcts, unstable angia and cardiovascular events. Heparin treatment can be inhibited by platelets activated by the F_c fragment of antibodies developed to the antigen of heparin-platelet factor 4. A corresponding inactivation of Refuldan is not known.

Lercanidipine (antihypertensive) (153-156)

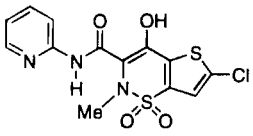
Country of Origin:	Italy
Originator:	Recordati
First Introduction:	Netherlands
Introduced by:	Byk Gulden
Trade Name:	Lerdip
CAS Registry No.:	100427-26-7
Molecular Weight:	648.20



Lerdip was launched in the Netherlands for hypertension. It is prepared in four steps, the last of which is a Hantsch reaction of 1-[(3,3-diphenyl)-N-methylpropylamino]-2-methyl-2-propyl-2-(3-nitrobenzylidene)acetoacetate with methyl 3-aminocrotonate. This compound was designed to be very lipophilic which imparts the drug with a gradual onset and a long duration of action as a result of prolonged exposure to receptors by partitioning into membranes. It is an antagonist of L-type calcium channels with no activity in smooth muscle cells, is tissue selective, lacks any myocardial contractility impairment, has no neuroendocrine activation, and has negligible affinity for neurotransmitter receptors such as α_1- and α_2-adrenergic receptors. While sold as a racemate, the calcium channel activity is found in the (S)-isomer with the (R)-isomer being 2-orders of magnitude less active. It has an increased cardiac contractility index which is much more than Nitrendipine or nifedipine. The good selectivity causes a reduction in blood pressure with no negative inotropic effects.

Lornoxicam (NSAID) (157-162)

Country of Origin:	Norway
Originator:	Nycomed Amersham
First Introduction:	Denmark
Introduced by:	Nycomed Amersham
Trade Name:	Xefo
CAS Registry No.:	70374-39-9
Molecular Weight:	371.83

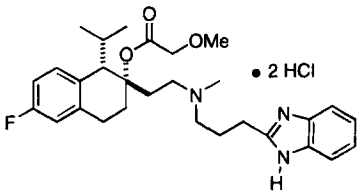


Xefo, a member of the oxicam family of nonsteroidal antiinflammatory agents, was launched in Denmark for mild to moderate pain and inflammation. A seven step

synthesis, beginning with 2,5-dichlorothiophene, provides access to this compound. It inhibits prostaglandin synthesis at the level of cyclooxygenase (Cox1:Cox2 = 0.6) but does not inhibit 5-lipoxygenase. Xefo did not increase serum levels of pepsinogen I (an indicator of gastric mucosal status) and readily penetrates perivascular interstitial spaces including synovial fluid. It is as effective as morphine, meperidine and tramadol in relieving post operative pain and as efficient as other NSAIDs in relieving the symptoms of osteoarthritis and rheumatoid arthritis. It is 100 times more potent than Tenoxicam in inhibiting PGD_2 and more active than indomethacin (6x) or piroxicam (10x) in preventing arachadonic acid influenced lethality in mice. Xefo has inhibitor effects on spinal nocicceptive processing presumably via release of endogenous opiods and evidence suggests it is good for migraine.

Mebefradil Hydrochloride (antihypertensive) (163-171)

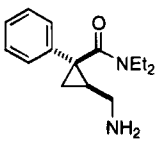
Country of Origin:	Switzerland
Originator:	Roche
First Introduction:	US
Introduced by:	Roche
Trade Name:	Posicor
CAS Registry No.:	116666-63-8
Molecular Weight:	568.56



Posicor was launched in Argentina, Azerbaijan, Brazil, the Chile, Czech Republic, Denmark, Germany, Ireland, Kazakhstan, the Netherlands, Norway, Peru, the Philippines, Switzerland, Thailand, Turkmenistan, the UK, the US and Venezuela for use in hypertension. It can be assembled in five steps from 4-(1-benzyloxy-N-methylformamido)butyric acid with o-phenylenediamine and isobutylchloroformate. Posicor binds to calcium channels in a manner that overlaps the Verapamil, SR-33557 and Diltiazem binding site without effecting the dihydropyridine binding site. Thus it blocks a wide variety of calcium channels with selectivity for T-type followed sequentially by L-type. There is also some calcium agonist activity along with sodium and potassium channel activity. It is chemically distinct from the other calcium antagonists (first member of a new catagory of tetralol calcium antagonists). It has the ability to lower heart rate with no negative inotropic effects and is as effective as Amlodipine and more effective than Diltiazem for the treatment of mild to moderate hypertension. Serious drug interactions are possible because Posicor inhibits both CYP2D6 and CYP3A4.

Milnacipran (antidepressant) (172-178)

Country of Origin:	France
Originator:	Pierre Fabre
First Introduction:	France
Introduced by:	Pierre Fabre
Trade Name:	Ixel
CAS Registry No.:	92623-85-3
Molecular Weight:	245.35

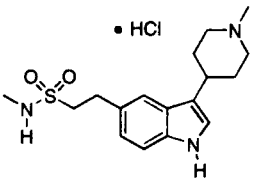


Ixel was launched in France as an antidepressant. There are several synthetic routes, the shortest of which is five steps using benzyl cyanide as the starting material. It is a specific serotonin and noradrenaline reuptake inhibitor (SNRI). This dual mechanism of action makes it superior to selective serotonin reuptake inhibitors (SSRI) like fluoxetine and fluvoxamine. Ixel has no significant effect on postsynaptic

receptors, very limited effect on cardiac function, and no quinidine-like arrhythmal effects. It has a good side effect profile with lower incidence of anticholinergic-like side effects, less sedation due to histamine H_1-receptor binding, and a lack of α_1-adrenoceptor antagonism. Ixel has a short half-life (7 hr) with no active metabolites. It is not metabolized by CYP450 therefore drug interaction is unlikely. It is superior in the treatment of serious depression with no need to titrate drug dose.

Naratriptan Hydrochloride (antimigraine) (179-183)

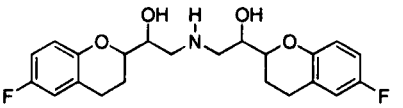
Country of Origin:	UK
Originator:	Glaxo Wellcome
First Introduction:	UK
Introduced by:	Glaxo Wellcome
Trade Name:	Naramig
CAS Registry No.:	143388-64-1
Molecular Weight:	371.93



Naramig was launched in Germany, Sweden and the UK for use in migraine. It is chemically available via a number of related synthetic routes all having about three steps starting from 5-bromoindole. It is a new serotonin $5\text{-}HT_{1B/1D}$ receptor antagonist with modest affinity for $5\text{-}HT_{1a}$ and very weak affinity for $5\text{-}HT_3$ receptors. It has little or no affinity for a wide range of non-serotonin receptors including α- and β-adrenoceptors, dopamine, neurokinin NK_1, and opiate receptors. It mediates vasoconstriction in cerebral vasculature (extra cerebral intracranial vessels), reduces neurogenic inflammation, and inhibits responses mediated by the trigeminal nerves. It has a 6- and 3-fold greater affinity for $5\text{-}HT_{1B}$ and $5\text{-}HT_{1d}$ receptors, respectively, than sumatriptan which translates to a 2-3 fold increase in potency. The reoccurance of headache was less compared to sumatriptan, zolmitriptan and rizatriptan. Naramig had no clinical effects on blood pressure or heart rate, had a long duration of action with very good tolerability, and has high oral bioavailability.

Nebivolol (antihypertensive) (184-188)

Country of Origin:	US
Originator:	Johnson & Johnson
First Introduction:	Germany
Introduced by:	Menarini
Trade Name:	Nebilet
CAS Registry No.:	99200-09-6
Molecular Weight:	405.44

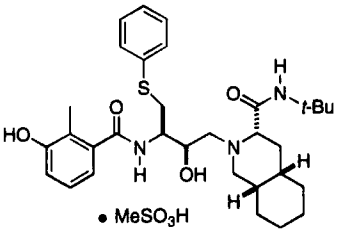


Nebilet was launched in Germany and the Netherlands as an antihypertensive agent. It is prepared by a five step route starting with 6-fluoro-4-oxobenzopyran-2-carboxylic acid. It is a selective β_1-adrenergic receptor antagonist and is 50 times less potent at β_2-receptors. Vasodialating properties occur via the nitric oxide pathway. While the L-isomer is more potent than the D-isomer, the racemic mixture is necessary for optimal activity. Upon administration, an immediate fall in blood pressure occurs, it improves both left ventricular systolic and diastolic function, and lowers peripheral blood resistance. Nebilet does not influence insulin sensitivity nor lipid profile comparible to atenolol. Doses of 50 mg of atenolol and 10

mg of Nebilet are equipotent but Nebilet has a longer duration of action due to an accumulation of the drug and increasing plasma levels of active metabolites.

Neflinavir Mesylate (antiviral) (189-195)

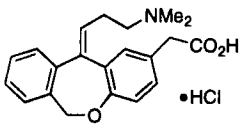
Country of Origin:	US
Originator:	Agouron
First Introduction:	US
Introduced by:	Agouron
Trade Name:	Viracept
CAS Registry No.:	159989-65-8
Molecular Weight:	663.89



Viracept was launched in the US as an orally-available, non-peptidic HIV protease inhibitor. It is prepared in an eight step synthesis beginning with N-(benzyloxycarbonyl)-L-serine β-lactone. The HIV protease inhibitory activity blocks the processing of *gag* and *gag-pol* polyproteins that are required for viral maturation. It was discovered based on an experimentally derived 3D-structure of HIV-1 protease. Viracept has a high oral bioavailability, lacks toxicity, and has a resistance profile different from other protease inhibitors. Viracept with ziduvudine and lamivudine generated a 98% mean reduction from baseline in viral load after 24 weeks compared to ziduvudine and lamivudine alone. Viracept also had better CD4 counts and is available in adult and pediatric formulations.

Olopatadine Hydrochloride (antiallergic) (196-204)

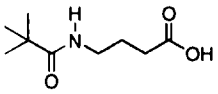
Country of Origin:	Japan
Originator:	Kyowa Hakko
First Introduction:	US
Introduced by:	Alcon
Trade Name:	Patanol
CAS Registry No.:	140462-76-6
Molecular Weight:	373.88



Patanol was launched in the US for use in allergic conjunctivitis. It can be prepared in five steps from the sodium salt of *p*-hydroxyphenylacetic acid methyl ester with phthalide. It has a fast onset of action with a long duration of action (due to slow dissociation kinetics) that combines the ability to prevent human conjunctival mast cell mediator release with selective H_1-receptor antangonistic activity (greater H_3:H_1 selectivity than H_2:H_1 selectivity). In addition, Patanol had no inhibition of 5-lipoxygenase, PAF acetyltransferase and thromboxane synthase while interfering with phospholipase A_2. It has a presynaptic inhibition of tachykinin release and inhibits bronchial sensory nerves through activation of small conductance calcium activated potassium channels. It was more potent than ketotifen and terfenadine and was effective at inhibition of PAF, LTC_4 induced conjunctivitis and TXB_2 production. It does not accumulate in the CNS, has a low affinity for H_1-receptors in the brain, and significantly inhibits allergen induce sneezing. Patanol was more effective in conjuctival than in corneal or the trabecular meshwork cells.

Pivagabine (antidepressant) (205-212)

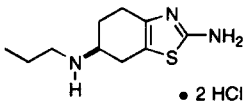
Country of Origin:	Italy
Originator:	Angelini
First Introduction:	Italy
Introduced by:	Angelini
Trade Name:	Tonerg
CAS Registry No.:	69542-93-4
Molecular Weight:	187.24



Tonerg was launched in Italy for treatment of acute and post traumatic stress syndrome and "burnout" syndrome. It inhibits hypothalamic release of corticotropin-releasing factor (CRF) and effectively antagonizes the activation of CRF induced stress (neuroendocrine, autonomic and behavior responses). Tonerg is an atypical antidepressant because it does not involve the monoaminergic system and differs in effect from benzodiazepines (complete opposite effect of Diazepam). The entire molecule is responsible for activity. It is lipophilic, orally bioavailable, and has a half-life of 4-6 hr. Tonerg has a positive effect on sleep disorders by reducing insomnia, improving anxiety by 20% and depression by 23%.

Pramipexole Hyrdochloride (anti-Parkinsonian) (213-220)

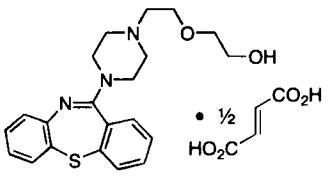
Country of Origin:	Germany
Originator:	Boehringer Ingelheim
First Introduction:	US
Introduced by:	Pharmacia & Upjohn
Trade Name:	Mirapex
CAS Registry No.:	104632-25-9
Molecular Weight:	284.25



Mirapex, an non ergot derivative, was launched in the US for treatment of Parkinson's disease. It can be prepared by two related routes, 5 steps and 3 steps, both involving an optical resolution with L-(+)-tartaric acid to afford the (S)-isomer. Mechanistically, it is a presynaptic dopamine D_2 autoreceptor agonist which also activates D_3-receptors. It is well absorbed from the GI and excreted in the urine essentially unchanged with a half-life of 8-12 hr. Mirapex had favorable effects on the symptoms without levodopa for patients with advanced PD and allowed a 27% reduction in use of levodopa in combination therapy. It had an adverse events profile similar to other dopamine agonists and in advanced patients reduced the mean "on-off" hr/day from 6 to 4. It was safe but not efficacious in all patients.

Quetiapine Fumarate (neuroleptic) (221-230)

Country of Origin:	UK
Originator:	Zeneca
First Introduction:	UK
Introduced by:	Zeneca
Trade Name:	Seroquel
CAS Registry No.:	111974-72-2
Molecular Weight:	441.54

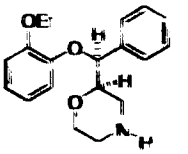


Seroquel was launched in the UK as an atypical antipsychotic agent for schizophrenia. It can be prepared in four steps from dibenzo[b,f][1,4]thiazepin-11(10*H*)-one. Seroquel is structurally similar to Clozapine with selectivity for the limbic region. It is a moderate dopamine receptor antagonist with greater affinity for D_2 over

D_1 and D_4, and greater affinity for 5-HT_{2a} than for D_2-receptors, although there is some ambiguity in the literature. Additionally, Seroquel is a strong antagonist of histamine H_1-receptors, has high affinity for α_1- and α_2-receptors with weak muscarinic affinity, and no benzodiazepine receptor affinity. Seroquel is 100% bioavailable, but first pass metabolism gives rise to 20 metabolites via CYP3A4, and has a half-life of 6 hr. It is as effective as Haloperidol and effective in treating both positive and negative symptoms of schizophrenia with low EPS effects.

Reboxetine (antidepressant) (231-239)

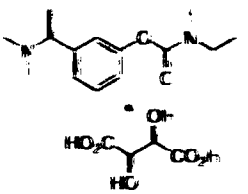
Country of Origin:	Sweden
Originator:	Pharmacia & Upjohn
First Introduction:	UK
Introduced by:	Pharmacia & Upjohn
Trade Name:	Edronax
CAS Registry No.:	71620-89-8
Molecular Weight:	313.40



Edronax was launched in the UK as an antidepressant. It can be prepared in 9 steps from cinnamyl alcohol. It is a selective noradrenaline reuptake inhibitor, therefore, the "central serotonergic syndrome" should not occur. Edronax provided effective treatment in the short and long term with efficacy in severe depression. It had advantages in social functioning by increasing energy, interest and motivation. Edronax was as effective as imipramine, desipramine and fluoxetine but more effective than fluoxetine in severe depression. It had minimal pharmacological impact on psychomotor and cognitive function with low toxicity. There was no MAO-A inhibition. It induced the down regulation of β-adrenergic receptors in rats and had no affinity for α-adrenergic, muscrinic cholinergic receptors. The (S,S)-(+)-isomer had a half-life of 13 h and was more potent than the (R,R)-(-)-isomer.

Rivastigmin Tartrate (anti-Alzheimers') (240-245)

Country of Origin:	Switzerland
Originator:	Novartis
First Introduction:	Switzerland
Introduced by:	Novartis
Trade Name:	Exelon
CAS Registry No.:	129101-54-8
Molecular Weight:	250.34

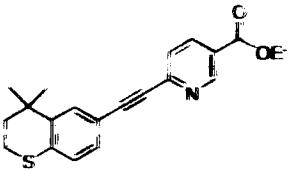


Exelon was launched in Switzerland for mild to moderately severe Alzheimer's disease. It is assembled in two steps from 3-[1-(dimethylamino)ethyl]phenol and N-ethyl-N-methyl carbamoyl chloride followed by resolution with (+)-D-di-O,O'-(p-toluoyl)tartaric acid. Exelon is a centrally selective and long lasting drug with anti-cholinesterase activity that facilitates cholinergic transmission in the cortex and hippocampus (targets for AD). It is also a pseudo-irreversible carbamate acetylcholinesterase inhibitor and normalizes impaired choline acetyltransferase activity. Increased extracellular levels of acetylcholine with no change in norepinephrine, serotonin, dopamine and their metabolites was also observed. While it has a half-life of 2 h, Exelon was able to inhibit the enzyme for 10 h due to a slow dissociation. It was safe and well tolerated in that there was no cardiac toxicity, no blood pressure changes, and no organ toxicity. A 3 mg dose resulted in a

30-40 % inhibition of brain acetylcholinesterase with minimal inhibition of peripheral enzyme, erythrocyte or plasma butylcholinesterase.

Tazarotene (antipsoriasis) (246-252)

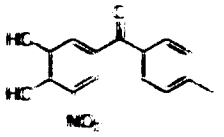
Country of Origin:	US
Originator:	Allergan
First Introduction:	Germany
Introduced by:	Allergan
Trade Name:	Zorac
CAS Registry No.:	118292-40-3
Molecular Weight:	351.46



Zorac was launched in Germany, Ireland, the UK, Canada and the US for psoriasis and acne. It is prepared in five steps beginning with a thiophenol displacement on 3-methyl-2-butenylbromide and acid catalyzed ring closure. Zorac normalizes abnormal keratinocyte differentiation and proliferation, and reduces expression of inflammatory markers. It has a half-life of 20 min in which it is converted to tazarotenic acid (half-life of 18 h), the biologically active form with receptor selectivity for the retinoid acid receptors (RAR). Structurally, it is a first generation conformationally restricted form of vitamin A and has affinity for the RARβ > RARγ >> RARα receptors with no affinity for the RXR receptors. It also up regulates the TIG1 and TIG2 genes with no rebound effect.

Tolcapone (anti-Parkinsonian) (253-259)

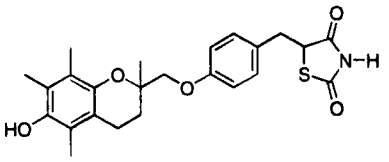
Country of Origin:	Switzerland
Originator:	Roche
First Introduction:	Germany
Introduced by:	Roche
Trade Name:	Tasmar
CAS Registry No.:	134308-13-7
Molecular Weight:	273.24



Tasmar was launched in Argentina, Austria, Canada, Denmark, Finland, Germany, Ireland, Sweden, Switzerland, the UK and Uruguay as an adjuvant to levodopa therapy for Parkinson's disease. It is synthesized in five steps from 4-bromotoluene and 4-(benzyloxy)-3-methoxybenzaldehyde. Tasmar is a peripherally selective, reversible, orally-active catechol-O-methyltransferase (COMT) inhibitor. This enzyme catalyzes the conversion of biologically active levodopa to the inactive 3-O-methyl form. The metabolite accumulates and contributes to the observed "wearing-off" phenomenon. It also competes for transport into the brain thus attenuating the activity of levodopa. Tasmar is well tolerated and its oxidized metabolite also inhibits COMT. In the presence of Tasmar, levodopa plasma levels increased which allowed for reduction in the levodopa dosage. It also increased L-DOPA and dopamine levels in the CSF. It may also have antidepressant activity and its antioxidant properties partially restored memory deficits.

Troglitazone (antidiabetic) (260-268)

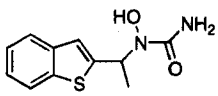
Country of Origin:	Japan
Originator:	Sankyo
First Introduction:	Japan
Introduced by:	Sankyo
Trade Name:	Rezulin
CAS Registry No.:	97322-87-7
Molecular Weight:	441.54



Rezulin was launched in Japan, the UK (subseqently withdrawn) and the US for treatment of type II diabetes. Two approaches, four steps and six steps, converge on 6-acetoxy-2-(4-aminophenoxymethyl)-2,5,7,8-tetramethylchroman which can be elaborated in two steps to Rezulin. It is the first of a new class of thiazolidenediones for NIDDM that reduces glucose concentrations without effecting insulin secretion. It binds to peroxisome proliferator-activated receptor gamma (PPARγ) thus activating this nuclear receptor which then influences carbohydrate metabolism. This is accomplished by increasing insulin sensitivity by upregulating glucose transporter (Glut1 and/or Glut4) expression without affecting the number or affinity of insulin receptors. There is also an increase in hepatic glycogen synthase activity which enhances glucose utilization and a reduction in hepatic gluconeogenesis by inhibiting fructose-1,6-bisphosphatase. Pancreatic islet cell destruction is prevented. It reduces serum triglycerides because PPARγ causes fibroblasts to differentiate into adipocytes and does not activate RARα. It has a half-life of 9 h and is metabolized into three compounds having no activity.

Zileuton (antiasthma) (269-277)

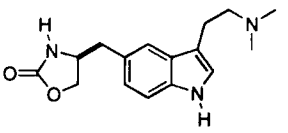
Country of Origin:	US
Originator:	Abbott
First Introduction:	US
Introduced by:	Abbott
Trade Name:	Zyflo
CAS Registry No.:	111406-87-2
Molecular Weight:	236.29



Zyflo was launched in the US for chronic asthma. It can be prepared in three steps from 2-acetylbenzo[b]thiophene. Zyflo is a reversible direct inhibitor of 5-lipoxgenase that is orally-active. It was able to effect a 70-100 % reduction in LTB_4, LTE_4, LTD_4 and LTC_4. Zyflo has no effect on myeloperoxidase activity, neutrophil degranulation, mast cell histamine release or phospholipase A_2 activities. It did not inhibit cyclooxygenase as witnessed by the formation of TXB_2. It significantly attentuated asthmatic response to cold dry air, inhibited exercise-induce bronchoconstriction, and attenuated induced bronchospasms. Zyflo has antiinflammatory effects as witnessed by a decrease in edema, mucus production and cellular infiltration. It had a bronchodilatory effect within 2 h and increased spirometry results by 18%.

Zomitriptan (antimigraine) (278-285)

Country of Origin:	UK
Originator:	Zeneca
First Introduction:	UK
Introduced by:	Zeneca
Trade Name:	Zomig
CAS Registry No.:	139264-17-8
Molecular Weight:	287.36



Zomig was launched in Germany, Denmark, Sweden and the UK for use as an antimigraine agent (with and without aura). It can be prepared by three related routes of 5 to 7 steps starting from L-4-nitrophenylalanine. Zomig is a 5-$HT_{1D/1B}$ receptor agonist (10 fold ratio) with modest (< 100 x) affinity for 5-HT_{1A} and 5-HT_{1F} receptors. It has no affinity for other serotonin receptors or receptors of other neurotransmitters. It has a novel dual action mechanism: centrally it acts on the trigeminal nucleus caudalis and peripherally is acts on the trigeminovascular system. Zomig was effective in treating headaches and nonheadache (photophobia, phonophobia and nausea) symptoms. It was 2-3 times more potent than sumatriptan and is metabolized to three compounds, one of which is 2-8 times more active than the parent. It caused a 40-50% decrease in headache after 1 h and a 73-77% after 4 h. There was a 30% reoccurance of headache but 90% effective treatment with a second dose. It blocks neurogenic inflammation by inhibiting release of peptides, causes vasoconstriction, and inhibits neuronal depolarization at peripheral sites in the cranium. It is 40% bioavailable and a 10 time theraputic dose showed no safety concerns.

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Chapter 33. Gender Based Medicine

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Introduction - A new era of clinical medicine that embraces and understands sex differences in drug response, metabolism and safety has dawned. This chapter explores the origins and implications of this new era. Until recently, there has been a paucity of data on gender differences in most diseases or disabilities, with the exception of areas that are specifically sex linked, e.g., contraception. Consequently, there is limited information about the influence of gender on most health conditions. There is a lack of data on sex=gender differences for a number of historical reasons (1). Typically, animal models of different disease states have involved only male animals, in part due to the complicating effects of the female reproductive cycle. The therapeutic agents that are identified by these models are not, in principle, fully "bisexual". Thus, the role of female reproductive hormones and metabolism on drug effects in animal models has remained unexplored.

Before 1985, women were rarely included in clinical trials. The thalidomide experience caused great concern about exposing women of childbearing potential to the possible teratogenic effects of investigational agents (2). This resulted in the exclusion of women from Phase I and Phase II, and often from Phase III clinical trials. In addition to the exclusion of women of childbearing potential in clinical trials, drug trials were based on the belief that male physiology was the implicit normal standard for studying the etiology, pathology and responses to new therapeutics. This provided the clinical researcher with a sexually homogenous population; the results from these trials were thought to be reliably and safely generalized to females (one sex fits all approach). Under this viewpoint, female drug response was explored only to the extent that female systems differed from the implicit norm (male physiology). Thus, gender based medicine was focused on conditions unique to the female reproductive life cycle. Because of this, gender based pharmacokinetic effects such as differential rates of absorption, excretion and metabolism that could have major implications for clinical trial design was also overlooked.

Today, the routine practice of exclusion of women from clinical trials has ended, due to the 1993 FDA and the NIH guidelines for the inclusion of women and minorities in clinical trials (3). We now expect that gender analysis will be performed routinely to identify potential differences in drug actions or efficacy between the sexes. Thus, the "gendercentric age" of drug investigation seems to be ebbing as investigators more fully appreciate the complexity of gender based drug responses. The recognition of gender differences in health and diseases should provide important insights into disease diagnosis, treatment and prevention for both men and women. This chapter will review gender based differences with an emphasis on pharmacokinetic studies, as well as gender specific manifestations of selected disease states.

PHARMACOKINETICS, PHARMACODYNAMICS AND DRUG METABOLISM

The pharmacokinetics of a drug and pharmacodynamics can be influenced by many factors such as gender, metabolic phenotype, exposure to other drugs and body weight and composition (4). Men and women, on average, differ in body size and composition, and metabolism and this may affect the efficacy and safety of a drug. For example, if a drug is administered on the basis of a fixed dose, an average female will receive a higher dose per kg than the average male. For drugs with a wide therapeutic index, this fixed dose dosing regimen is not important. If the

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therapeutic index is narrow, these differences become clinically significant. This warrants a detailed study of the sex differences for drugs with either steep dose responses or narrow therapeutic indices. Women have a lower ratio of lean body mass to adipose tissue, and this difference in body composition may affect drug disposition (5,6). Lipophilic drugs such as the benzodiazepines, on average, have a greater volume of distribution in women, and this can alter their therapeutic dose. Sex differences also can play an important role in drug absorption; for example, females show slower gastric emptying and gastrointestinal transit time than males and this is thought to directly correlate with the level of sex hormones (7-11).

Antipsychotic drugs have been extensively reviewed for gender specific pharmacokinetic and pharmacodynamic differences (5,12,13). For example, fluphenazine was seen to have higher plasma levels in women despite comparable dosing, and similar age and weight characteristics for men and women (12). Treatment with fluspirilene, a long-acting injectable neuroleptic agent, showed that women required significantly lower doses than men in an age and weight matched study (13). Orally administered thioxthexene had significantly higher clearance in men than women and this clearance did not necessarily correlate with body weight (14). The pharmacokinetic profile of sertindole, a new selective antipsychotic, was investigated for age and gender effects. After multiple dosing, Cmax (mean peak plasma concentration) and AUC (area under the plasma concentration curve) were higher in females than males of similar age, implying that there may be a higher extent of absorption or a dependence of sertindole clearance on lean body mass (15). Pharmacokinetic studies of venlafaxine, a new generation antidepressant which acts specifically by inhibiting the reuptake of serotonin and norepinephrine, revealed that gender based differences did not occur and therefore dosage adjustments were not necessary (16-18). This illustrates an interesting point. Simpler dosing is a competitive advantage in the marketplace. In the future, drugs may be selected on the basis of a lack of gender based dosing effects.

Drugs are often metabolized in the liver through various enzymatic pathways. The pharmacokinetics of hepatically metabolized drugs, such as the benzodiazepines, have been extensively studied and clearly demonstrate the complex role of gender on drug metabolism (5,19). Benzodiazepines that are metabolized primarily through conjugation, such as temazepam and oxazepam, are cleared faster by men than women (20-22). In contrast, alprazolam (23) and diazepam (21) are metabolized primarily via an oxidative mechanism and are cleared faster by women than men. Finally, no sex differences were observed with nitrazepam (24), which is metabolized via reduction of its nitro group. These studies highlight the fact that sex differences influence the metabolic pathway which can be seen even within drugs of the same pharmacological class and which are structurally similar.

The cytochromes P450 (CYP) are a superfamily of hemoproteins that consist of a set of isozymes that are intimately involved in the oxidative metabolism of drugs in the liver. This area of research has recently been reviewed in detail (25). In mammals, at least 14 P450 gene families have been identified. However, only CYP1, CYP2 and CYP3 are thought to be responsible for most hepatic metabolism of drugs. The CYP3A4 subfamily is the most abundant CYP in the human liver and is responsible for metabolism of many drugs such as cyclosporin, quinidine, erythromycin, dapsone and lidocaine (19,26). Data from various drug studies (tirilazad (27), erythromycin (28), diazepam (21)) suggest there is a significant gender difference in CYP3A4 activity, with females having greater CYP3A4 activity than males. Drugs metabolized by CYP3A4 are extensively cleared in women, whereas drugs cleared by other isozymes of P450, are usually cleared faster by men (19). The gender specific differences in CYP3A4 activity are thought to be mechanistically related to the female sex hormones, estrogen and progesterone, which may regulate CYP3A4 activity at the level of gene expression (19). From this information, one can anticipate gender differences in pharmacokinetics for drugs metabolized by the P450 pathway. However, there are examples such as ranitidine, which is metabolized by P450's, where no gender differences were seen (29). Finally, some drugs are

metabolized solely through conjugation and whereby gender differences on elimination have been seen (21,22,30-33).

Cardiovascular agents have also been well studied for gender specific pharmacokinetic and pharmacodynamic differences. Verapamil (34) is metabolized through the CYP3A4 pathway and shows gender differences in clearance rates. The metabolic rate was 20% greater in women than men. Propranolol is administered as a racemic mixture and is eliminated faster in men than women (35-38). The steady state concentration of the drug is greater in women than men. Propranolol is metabolized by three different pathways: ring oxidation mediated by CYP2D6, side-chain cleavage by CYP1A and CYP2C, and, glucuronidation mediated by glucuronosyl transferases (19,38). The sex effect on propanolol metabolism appears to be metabolic pathway specific (38). Side-chain cleavage and glucuronidation were faster in males than females while ring oxidation (CYP2D6 pathway) was seen to be at parity in both sexes. In the case of propranolol, the clearance of unbound S-propranolol is significantly greater than the R-isomer in females but not in males (36). Therefore, the average concentration of the R-isomer at steady state is greater than that of active S-isomer in females. There was no difference between the sexes in unbound clearance of the S-enantiomer.

Common gender-specific responses to drug treatments are almost certainly related to various physiological states that are specific for females. Menopause, pregnancy, lactation and hormonal changes that occur during the menstrual cycle are known to be clinically important factors that can influence drug metabolism. One of the most drastic physiological changes that the female experiences is the menopause. Many women undergo a slow transition from normal menstrual cycles to irregular menstrual cycles to the lack of menstrual cyclicity. Menopause is defined as a cessation of ovarian function in which the ovary is no longer capable of secreting steroids, the body's main source of estrogen and progesterone. On average, the postmenopausal state will comprise greater than one-third of the life span of the average female. Menopause is likely to be a contributing factor to age-related changes in drug metabolism when the age-related change occurs in females and not in males (39,40). An example of an age-associated change in drug metabolism was reported with alfentanil. Females showed an inverse correlation between alfentanil clearance and age (41,42). However, no change could be observed in males. Also, total and unbound clearance of prednisolone was lower in postmenopausal women than in premenopausal women (43). The non-steroidal anti-inflammatory agent, piroxicam, had decreased clearance in elderly women vs. young women, with no differences observed in men. Other contributing factors that could alter drug metabolism during the menopause are hormone replacement therapy (44,45) or the selective estrogen receptor modulator (SERM), raloxifene, which may be utilized to alleviate some of the unpleasant symptoms of menopause, as well as reduce the risk of osteoporosis (46). Because of the increasing popularity of hormone replacement therapy, and the recent introduction of SERMs as therapeutic agents, this is an area that needs further exploration.

Pregnancy is another physiological state that can profoundly affect the pharmacokinetic profile of the drug. During pregnancy, the placenta can secrete large amounts of hormones, which can further confound the drug metabolism profile, although during pregnancy, drug therapy is typically minimized, there are diseases, such as epilepsy, where continuation of drug therapy is necessary, and the enhanced clearance of antiepileptic agents has been reported (47,48). Dosages of common antiepileptic drugs, such as phenytoin, phenobarbital and carbamazepine had to be increased in the majority of pregnant women to maintain therapeutic concentrations of the drugs (49).

The cyclic pattern of estrogen and progesterone secretion during the menstrual cycle may also contribute to alterations in drug metabolism (50-52). This is an area that has not been fully explored and is prime for further investigation.

One group of drugs which is in widespread usage by females that could alter the metabolism of other drugs is the oral contraceptives. Two major mechanisms appear to be responsible for the effect of oral contraceptives on drug metabolism. Both mechanisms appear to be related to hepatic function. Oral contraceptives have been reported to inactivate cytochromes P450 (53,54) and also increase the levels of glucuronosyl transferases. The inhibition of cytochromes P450 can reduce clearance of a co-administered drug, resulting in a higher plasma concentration and perhaps even result in toxicity, such as the cardiotoxicity seen with erythromycin. The clearance of two other drugs with a high potential for toxicity when overdosed (cyclosporin and theophylline) is decreased after oral contraceptive treatment (55,56). Many benzodiazepine tranquilizers and hypnotics are metabolized by hepatic microsomal hydroxylation and their metabolism is therefore at risk of being inhibited by the contraceptive steroids (57,58). Oral contraceptive administration has also been reported to reduce drug glucuronidation of clofibrate (30) and paracetamol (59), thereby increasing clearance and lowering plasma drug concentrations. Thus, it is possible that concentration of drugs with a narrow therapeutic index could shift to a non-efficacious or even a toxic level, depending on hormonal-induced changes in hepatic drug metabolism.

Although oral contraceptives in some instances can alter the efficacy of certain drugs, drugs that induce P450 function, such as phenobarbital (56), phenytoin (60) and rifampicin (61,62), can reduce the efficacy of oral contraceptives. Additionally, antibiotics such as cephalosporins, tetracyclines and penicillins have been reported to decrease the concentration of oral contraceptives, thus altering their contraceptive efficacy (56).

LESSONS FROM THE TIRILAZAD TRIAL

Tirilazad mesylate, a nonglucocorticoid 21-aminosteroid compound and a specific membrane lipid peroxidation inhibitor, is a classic example where differential metabolic effects between men and women were observed (63). Tirilazad is eliminated via oxidation by the CYP3A4 isozyme which is more important in females than males, thus the clearance is much faster in women than in men. In the European subarachnoid hemorrhage clinical trial, the outcome was a statistically significant improvement in males but not in females when a gender analysis was done. This differential response was linked to the fact that women metabolized the drug 60% faster than men; because of this, the fixed dose design led to an inappropriately low drug exposure in females (64). Consequently, in some countries, tirilazad was approved for the treatment of subarachnoid hemorrhage in men and not women. Some of these potential pitfalls could have been overcome if gender based pharmacokinetic studies had been undertaken in the earliest phases of clinical development, rather than employing a "gendercentric" approach.

AUTOIMMUNE DISEASES

Autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), myasthenia gravis and multiple sclerosis (MS) are largely diseases of young women. Women are disproportionately affected, and many times they also have more severe disease. The actual female/male ratio can range from 10:1 for SLE, to 4:1 for RA (65). How, in the face of the complexity of the etiology and pathologenesis of these autoimmune disorders, do sex-linked differences in susceptibility arise? Although the explanation for this increased susceptibility is unclear, sex hormones, genetic factors and environmental factors have been proposed to play a role. For example, gender-related differences in both humoral and cellular immune responses and the role of sex steroids have been reviewed (66). Epidemiological studies on the use of oral contraceptives have demonstrated a lower incidence of rheumatoid arthritis. Hormone replacement therapy during the perimenopause and menopause has also implicated a beneficial role of estrogen in RA. During pregnancy, when steadily increasing levels of estrogen and progesterone are found, the clinical symptoms of RA and MS are markedly improved (67). Based on these observations, clearly the increased distinct female susceptibility for these

autoimmune diseases is not exclusively linked to estrogen. Genetic linkage studies have confirmed the major histocompatibility complex (MHC) as an important contributor to human autoimmunity (68).

Type I diabetes is another autoimmune disease resulting from the destruction of the insulin secreting pancreatic beta cells. The non-obese diabetic (NOD) mouse is a diabetic model of autoimmunity with marked gender differences in disease symptoms. For example, pancreatic insulitis is observed in both sexes. However, overt disease expression occurs predominantly in females (85-95%) with less than 10% of male NOD mice developing hyperglycemia (69). Using the NOD mouse model as a model for gender controlled autoimmune expression, Faustman, *et.al.* have demonstrated an influence of gender on MHC class I expression and function. Recent studies have reported in female NOD mice a mutation in the MHC region that effectively cripples antigen processing in antigen-presenting cells (APC) (70). This defect inactivates two genes that are critical for antigen processing and maturation of lymphocytes. These immature APC's fail to teach T-cells what is self vs. non-self. These two MHC linked genes have been shown to be differentially regulated at the transcriptional level between female and male NOD mice (71). Interestingly, people with autoimmune diseases such as lupus, RA, MS and Type I diabetes also possess a similar defect in antigen presentation and immature APC (72).

MS, the most common autoimmune disease of the central nervous sytem, also has a skewed sex distribution: women are disproportionately affected with a ratio of 2:1 (73). One mediator that has been postulated to play a role in the pathogenesis of MS is nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS). Recently, gender related differences of iNOS expression and NO production in SJL mice with experimental autoimmune encephalomyelitis (EAE) were reported (74). EAE in SJL mice is an autoimmune demyelinating disease of the CNS which mirrors human MS, clinically, immunologically and histopathologically. When myelin basic protein-specific T-lymphocytes from female mice were adoptively transferred to female and male recipients, females developed EAE with an earlier onset and greater severity as compared to males (75). This increased severity of disease in females correlated with increased levels of iNOS mRNA, enzyme activity and NO. In contrast, when T-lymphocytes derived from male mice were adoptively transferred, severe EAE was not induced in female or male recipients, and there was no detectable level of iNOS activity or NO.

ACUTE NEURODEGENERATION

Acute neurologic disorders include traumatic brain and spinal cord injury, ischemic and hemorrhagic stroke and brain damage after cardiac arrest. Gender specific responses to experimental ischemia, and other sources of brain injury have been reported. For example, female gerbils following a 3h period of severe incomplete brain ischemia produced by unilateral carotid occlusion (UCO) were reported to have improved outcome (less CA, hippocampal and cortical cell loss) as compared to male gerbils (76). In this same model, an important role of reactive oxygen species/lipid peroxidation in postischemic neuronal necrosis has been documented. Significant vitamin E depletion occurs by 2h following reperfusion presumably due to post-ischemic lipid peroxidation with the reduced form of the endogenous lipophilic antioxidant vitamin E being utilized to quench postischemic peroxidative reactions. Following 3h of ischemia plus 2h of reperfusion, the levels of vitamin E in the ischemic hemisphere in male gerbils decreased by 43.5% while the female gerbils displayed a 4.2% decline. Moreover, estradiol and two principal metabolites of estradiol are potent inhibitors of lipid peroxidation (77). One can hypothesize that the increased postischemic survival and neuronal preservation observed in female gerbils in the 3h UCO model may be due to a protective antioxidant effect of endogenous female hormones.

In a rat model of thromboembolic stroke induced by photochemical irradiation of the carotid artery, female rats had smaller infarcts than male rats (78). In another model of focal stroke, the middle cerebral artery occlusion model (MCAO), female rats sustained smaller cortical and striatal infarcts than age matched male rats after 2 hours of ischemia (79). Interestingly, this observed gender difference in infarct volume was lost when female rats were ovarietomized. This advantage that was abolished by ovariectomy could be restored by chronic exogenous estrogen administration (80). Finally, estrogen administration in age-matched male rate also conferred neuroprotection following 2h of MCAO (81). The salvage of neural tissue in intact females is likely due to female sex hormones, most likely estrogen and perhaps progesterone. In fact, progesterone has been shown to be neuroprotective in transient focal ischemia in the male rat when administered pre ischemia or 2h post ischemia (82). Chronic exogenous treatment with 17-B-estradiol augmented residual cerebral blood flow during global cerebral ischemia in rabbits (83). Sex based differences in cerebral edema in a model of traumatic brain injury (TBI) have also been studied. Cerebral edema, or accumulation of water in brain tissue, is a serious complication following brain injury. In a model of cortical contusion injury, intact female rats had less cerebral edema than males, again suggesting a role for female sex hormones (84). There are beneficial effects of progesterone treatment on cerebral edema following TBI in both male and female rats when administered as late as 24h post TBI (85). In contrast, it was shown that pretreatment with estrogen exacerbated acute neurologic deficits following TBI, specifically in female gerbils, and yet improved outcome in male gerbils (86). Clearly, gender differences have been seen in experimental models of brain injury. The role and mechanisms by which female sex hormones attenuate brain damage is complex and not fully understood.

Clinical studies have reflected what has been observed in animal studies. The incidence of atherosclerotic vascular disease and stroke is lower in premenopausal women than men of the same age. However, after menopause, the incidence of cardiovascular and cerebrovascular events rapidly increases in women. These epidemiological studies have also provided evidence that female sex hormones confer vascular protection in ischemic heart disease and stroke (87).

Additionally, ERT (estrogen replacement therapy) was associated with decreased risk and later age of onset of Alzheimer's Disease (88). The mechanism of this neuroprotective effect of ERT is unclear. However, recent studies demonstrated that estrogen treatment enhanced synaptic sprouting in response to entorhinal cortex lesioning in mice (89). This is in concert with earlier observations that estrogen increased dendritic spines in hippocampal neurons (90)

CLINICAL IMPLICATIONS OF GENDER DIFFERENCES

The clinical significance of gender differences is yet to be fully realized. Sex differences in the pharmacokinetics of drugs with a wide therapeutic index may not always be clinically important since the magnitude of their response may not be large enough to warrant dosage adjustment. Sex difference responses in pharmacokinetics can be significant when determining the initial dose of drugs with a narrow therapeutic index. Pharmacodynamic differences in the response to drugs by males and females can be clinically meaningful both for drugs with a wide therapeutic index as well as for those with a narrow index. These gender differences can translate into a higher incidence of adverse reactions to drugs observed in women compared to men. For example, a large number of drugs from diverse classes such as antiarrhythmic drugs, antihistamines (terfenadine) and antibiotics (erythromycin) have the potential to induce a potentially lethal cardiac arrythmia, torsades de pointes (91). The risk of developing this arrythmia when taking these drugs is far greater in women than in men. The electrophysiological mechanism underlying gender differences in susceptibility to torsades de pointes is not known. One hypothesis is differences between males and females in cardiac ion channels that have been shown to result in differences in drug response (92). In fact, females possess a longer average QT interval of the electrocardiogram (ECG) than men. Furthermore,

in isolated rabbit hearts, sex hormones have been shown to have effects on cardiac repolarization (93). The QT interval of the ECG was prolonged with estrogen treatment. The dose of a drug may be different for a female, not just because of differences in body size, but also because of potential gender-induced physiological differences.

Conclusion - The "gendercentric" approach to the treatment of diseases with drug therapy and its potential limitations and consequences is now being recognized. This recognition is a first step in providing drug therapy that is appropriate for every patient, male or female. Clearly, further research is needed to elucidate the mechanisms underlying these gender specific differences.

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Chapter 34. Technology Providers and Integrators—a Virtu. Architecture for Drug R & D?

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Introduction - In this decade substantial pressures on the pharmaceutical industry th. have built up over many years finally resulted in dramatic change. Paradoxically, thes. changes involved both increased entrepreneurial activity in the biotechnology sector and mergers and acquisitions with concomitant down-sizing among the large pharmaceutical companies. The reasons for these pressures can be broadly given as:

Increased Costs Pharmaceutical R & D has increased in cost faster than inflation for over 20 years. R & D revenue spending world-wide increased from just over $5 billion in 1981 to $33 billion in 1995. Current estimates of the cost of bringing a new chemical entity to the market are around $500m (1).

Decreased Patent Life Increased development times have resulted in progressively decreased periods of patent protection for new drug entities. As a result, modern patent strategy defers the filing of new molecule (composition of matter) patents as long as possible, and extends the lifetime of protection by use of novel formulations and other methods. In part, these measures are also a response to the increasing competition from generics which result in an extremely rapid decline in the sales of an off-patent drug. A new pattern of biological target patenting is emerging which seeks to protect research activities.

Reimbursement In most countries, drugs are at least partly paid for by government, and drug pricing is a political issue. Companies need to demonstrate pharmacoeconomic arguments to justify their pricing strategy, but even so governments often take a narrow view as to the advantages for which the industry can charge.

Difficult Areas All the easy therapeutic areas have been addressed. We are left with the difficult ones. So-called 'me-too' drugs are poorly regarded by the regulatory bodies, which look for substantial clinical advantages with new drug applications.

These problems have brought with them a re-evaluation of the current *modus operandum* for pharmaceutical R & D. The historical belief in the advantages of the aggrandizement of research and development operations has been questioned, largely from the viewpoint of whether these economies of scale can adequately compensate for the loss of creative individualism that is the life-blood of success in this industry. There are very significant challenges ahead in the next decade.

At the heart of the problem facing new product introductions in the industry is risk. It is unique to pharmaceutical research insofar as no other industry has quite so many candidates to investigate before a product can appear. Traditional estimates suggest that only one in a thousand drugs that are ever synthesised and tested are ever developed to the stage of being introduced into man; and only one in ten of these is ever registered and marketed. Moreover, only one in three marketed compounds returns their R & D investment; this commercial risk is still insufficiently appreciated (2).

New Technologies - In an effort to decrease research times, great strides have been made in automated techniques, which can broadly be grouped into three types:

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- Genomics
- Combinatorial chemistry
- High throughput Screening

All of these can be said to rely to a greater or lesser extent on improvements in automation and information technology as enabling tools. Genomics includes a wide variety of early stage research tools for uncovering new biological targets and is applicable very early in the R & D process. It represents an additional stage in R & D, one that reveals in greater detail than before the complexity of biological systems. In many cases, the first information available is little more than a gene sequence; this needs to be followed by information on the protein and its importance in disease mechanism before a project proposal can be realistically begun. Such target validation may include a basic biological research program into the gene sequence of interest (e.g. knock-out transgenic animals), supported by some clinical evidence of the importance of the particular biological pathway or protein of interest—this is functional genomics, a foundation for future activities (2).

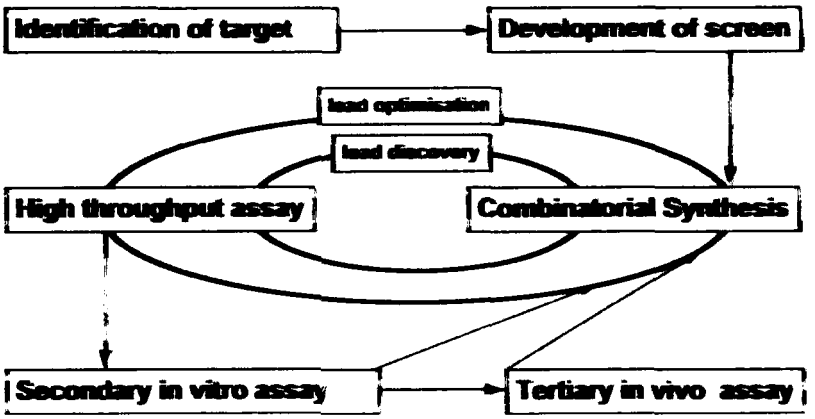

Figure 1 Iterative cycles in lead discovery and optimisation assisted by automated techniques

The second step of the product innovation process is increasingly reliant on the other two new technologies, combinatorial chemistry and high throughput screening. These work together, feeding off and supplying the needs of the other (Figure 1). In part, they apply the statistical principles of random screening, undaunted by the expectation of making and testing huge numbers of compounds in order to find good leads for a given target. For a new biological target, the discovery of a quality lead compound worthy of entry into an optimisation programme typically requires the synthesis and screening of around 100,000 diverse compounds (3). However, the modern lead discovery process differs from the old methods of random screening in the additional use of modern methods of computer analysis, enabling a more intelligent approach. These methods include prediction of molecular and physicochemical properties, so that the synthetic targets cover as much structural diversity as possible; data mining techniques to hunt for likely hits in huge databases based on a given pharmacophore; as well as more sophisticated methods of modelling and QSAR analysis. Combinatorial chemistry grew out of solid phase peptide techniques, although there are also combinatorial methods relying on solution phase chemistry too (4). The early enthusiasm for this technology in terms of number of compounds synthesised has given way to a realisation that quality is paramount. While the original work in this area was directed towards the production of substantial numbers of compounds in a mixture,

the recent trend has been for the synthesis of single compounds, and as the technology has become more widespread combinatorial chemistry providers supply chemical libraries, rather than technology. The principles of combinatorial chemistry have application in other areas beyond lead discovery. They can clearly be used for lead optimization, but they also can be used for process chemistry optimization, thereby accelerating route improvement for chemical scale up (5).

The trends in combinatorial chemistry have been complemented by the increased capacity in high throughput screening assays. New methods such as time resolved fluorescence and colorimetric reporter assays have theoretically enabled up to 100,000 single point assays per day. In practice, this number is substantially reduced by the inefficiency of machine setting up and breakdown. These new techniques to replace radiochemical methods offer advantages of increased sensitivity, reduced counting times and the generation of less waste. Miniaturised assays employ less biological material, require less chemical compound and can permit simultaneous counting of up to 1,536 wells per microplate, and beyond (6,7).

The value of combinatorial chemistry and high throughput screening is especially evident in the lead discovery step, both with regard to speed and predictability. Andersen consulting recently surveyed the R&D functions of ten pharmaceutical and biotechnology companies in Europe and the US, and produced an analysis forecasting substantial improvements in research productivity (8). Based on the participants' responses, the report suggested that lead identification times would come down from an average of 15 months in 1996 to just over 6 months in 2000. However, the impact of these new technologies in other parts of pharmaceutical research and discovery is also evident (Figure 2). Taken together, we have a situation in which the discovery phase of new product innovation is altogether shorter and more predictable in timescale.

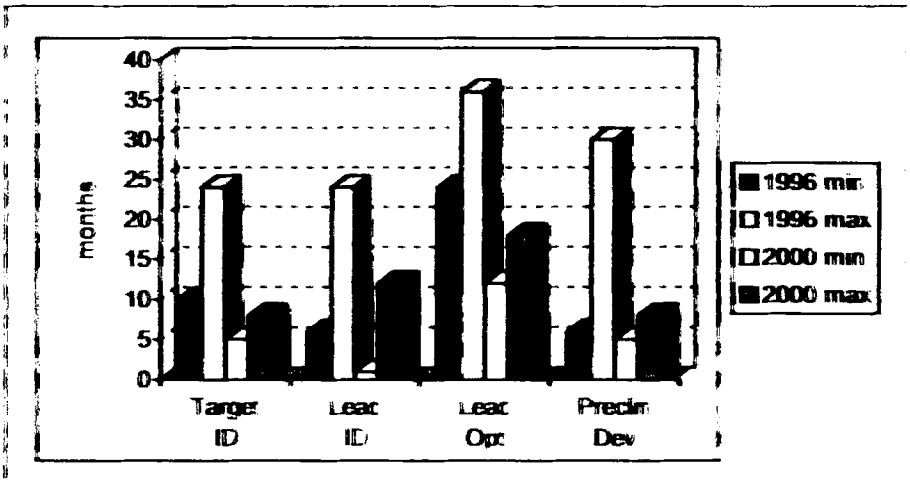

Figure 2 Minimum and maximum research and discovery times in 1996 and forecast for 2000 (data from *Scrip Magazine*, November, 35-38 (1997)).

Based on this analysis, a modern view of the overall process of innovation suggests that it is more appropriate to term it Research, Discovery and Development (R, D & D) (Figure 3). The research phase involves target validation and assay development. The discovery phase involves the lead identification and optimization stages which produce a candidate for entry into exploratory development. The lead optimization phase requires the close integration of biological and chemical resources; the biological effort is substantially more complex than that employed in the lead discovery phase. Beyond

simple screening assays, functional *in vitro* and *in vivo* tests are necessary, and increasingly high throughput assessments of absorption and metabolism are also used (9,10). In the future we can expect *in vitro* toxicological assessments will facilitate only the progression of compounds with the best profile into development. The speed of preclinical development indicated by the Andersen study is contingent in part on the selection of the easiest compounds to develop. Compounds with greater potency impose lesser demands on supply for developmental studies; compounds with favourable metabolic and/or chemical stability, solubility and morphological characteristics enable less complicated formulation and pharmaceutics packages (11).

The R, D & D paradigm, together with its possibilities for partnerships brings us to a consideration of the strategy for outsourcing in the pharmaceutical industry.

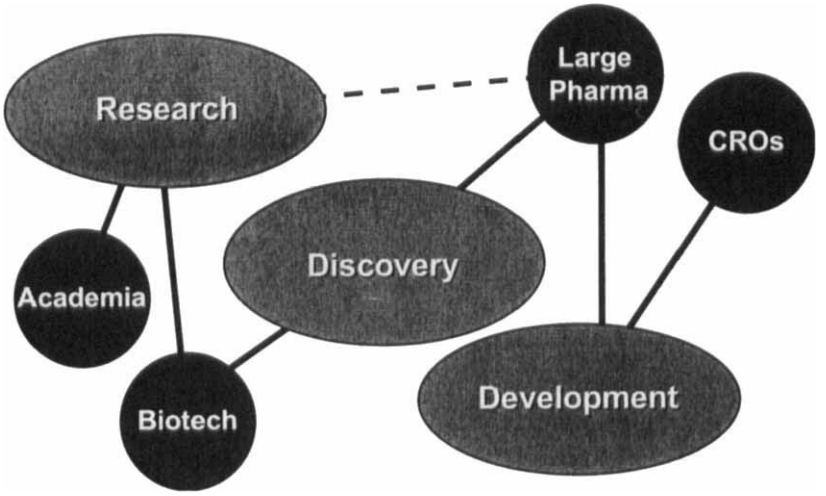

Figure 3 The R, D and D model for pharmaceutical innovation.

Philosophy Of Outsourcing - The Lehman brothers' report of 1996 looked at the economics of pharmaceutical R&D (12). They produced an estimate for internally developing a NCE of $205 million, whereas the cost for a totally outsourced development program was $40 million. From the viewpoint of the large pharmaceutical company, outsourcing of developmental activities will generally involve contract research organizations (CROs), which perform an increasing proportion of late stage pharmaceutical development. Some of the larger CROs now submit more new drug applications (NDAs) than many medium-sized pharmaceutical companies. Moreover, as the quality of expertise and the quantity of experience within these organisations increases, their utility for consultancy services also increases. It is expected that by 2000, 20% of pharmaceutical R&D budgets will be spent externally, up from a figure of 5% a decade earlier (13). Although as a sector, CROs have grown substantially, they have been in existence for a generation.

The possibilities for outsourced research have changed far more radically. Until recently, nearly all pharmaceutical research was conducted internally. In general, modern strategy now endorses research portfolios that include projects or technology partly provided extramurally by biotechnology companies and academic groups (an increasingly blurred distinction).

In-licensing early-stage research projects and development candidates are traditional activities for large pharmaceutical companies. However, the surge in new technology has generated an additional target for licensing. Much of the new

technology described above derives from new, small and medium sized enterprises (SMEs), and these organizations typically provide this technology to a number of clients. As the technology has matured, SMEs have been willing to work by contract rather than by research collaborations involving prior negotiation of intellectual property rights. These changes, in the existence of this technology within SMEs outside the traditional pharmaceutical industry, and more recently the opportunity to organise by contract, offers a highly flexible and adaptable strategy for modern drug discovery.

The argument for outsourcing is not entirely one-way (14). There are disadvantages as well as advantages. However, the historical reliance on almost entirely internal activities is rapidly diminishing. Partly, this is due to the increasing availability of quality research outside the large companies. In addition, other reasons start with the economic advantages indicated in the Lehman report, which were founded in part upon the savings due to changing the costing of studies from a fixed to a variable budgetary basis. One major fault of internal operations in a complicated project is the cost of delay. It is a factor not limited to pharmacautical development, delay is a significant problem in any multicomponent project. Delay causes consequential slippage in addition to the naked time loss of a problem affecting a single component. Internal studies will incur costs while the staff and equipment lie idle awaiting delayed studies to start. Externally contracted studies are not subject to this problem. The need to fill the resource at the contractual location falls to the contractor, who has a number of clients to look to (certainly more than the opportunity for work generated within even a large pharmaceutical company). This embodies the management theorist's adage that one should insource to the troughs and outsource to the peaks in any project.

The full range of further advantages of external collaboration or contraction can be summarised as follows:

Large company perspective	***Small (e.g. biotechnology) company perspective***
Better control on costs	Global marketing
Access to specific technology or expertise not present internally	Access to development skills
Access to such technology cheaper and quicker than internal alternative	Access to sales forces
Flexibility in disengagement from unsuccessful research; greater objectivity in making that decision	Security and management of risk
Small companies offer a more dynamic, flexible approach and better motivational environment for bright, enterprising scientists	Respectability • investors • future collaborations
Easier allocation of resources in a project with variable demand	Cash

Against these must be set the disadvantages of such a strategy taken from the large company perspective, as shown in Figure 4. Some of these advantages are more perceived than real. For instance, the oft-repeated problems of loss of control can be limited by effective communication and project management. Similarly geographical and cultural problems may not always be a problem, given that English is the predominant language of business and that electronic methods of communication over long distances and multiple time zones are evermore sophisticated.

Nevertheless, a common cause of recurring problems can be traced to unwillingness within the client organisation to devote sufficient resources to managing the collaboration. This may derive from a mistaken belief by senior management that

outsourced projects require little or no monitoring. One needs to compare the resources devoted to monitoring of clinical trials from within the medical departments, most of such trials having been conducted extramurally for many years Typically, an internal resource equivalent to around 20% of the level of the external manpower is required (15).

- Loss of control
- Cost
- Culture
- CROs
- Academia
- Time to agree contracts
- Intellectual property
- Co-ordination and expertise
- Instability
- Geographic distance, language & cultural barriers

Figure 4 Perceived disadvantages of collaborations

Architecture of R & D - Management of the risk associated with pharmaceutical R&D led throughout the 1980s and early 1990s to huge emporia devoted to science, in which the multitudinous activities were usually placed in one site. Glaxo Wellcome's monument to R & D, centralised at Stevenage in the UK, was completed in 1995 at a cost of practically $1 billion, and at the time was the second most expensive construction project in Europe.

One may conceptually draw these efforts as a single Doric column, in which scientific departments align in close proximity (Figure 5). This is the diagrammatic representation of what has become known in business analytical circles as the FIPCO (fully integrated pharmaceutical company). The interplay between disciplines has been enhanced by propinquity, while the effort required to facilitate integration and co-ordination was minimised by this structure. The vertical dimension of the column is indicative of the timescale of the efforts that need to occur during the course of the discovery and development of a drug, with research and discovery at the bottom being succeeded by development above.

Adaptation to difficult and lengthy R&D programs has required commensurate increases in the height and diameter of the column, representing the demonstrably greater resources, applied over longer times, required in the process. Substantial infrastructure costs are associated with these in-house R & D efforts, but changing the shape of the column is difficult without major efforts; its internal infrastructure is heavy and inflexible, and poorly suited to a rapidly changing scientific environment. When combinatorial chemistry replaces traditional chemical techniques, the parameters for laboratory design are dramatically different. When new drugs are sought from molecular biological investigation, the personnel and the laboratory areas need to be changed.

As technology has been sought externally, large pharmaceutical companies have devoted substantial funds in support of R&D, either in relation to technology, or research projects. The partnerships that have formed between these small companies and larger ones are linked in much the same way as the pier buttresses are linked to the main body of a gothic cathedral via the flying buttress (Figure 5). When originally designed in the Middle Ages, buttresses were constructed to enlarge the cathedral windows, admit more light and generally to magnify the space inside to create a more impressive space in which to worship. In order to achieve these aims, additional force from the outside was applied to prevent collapse.

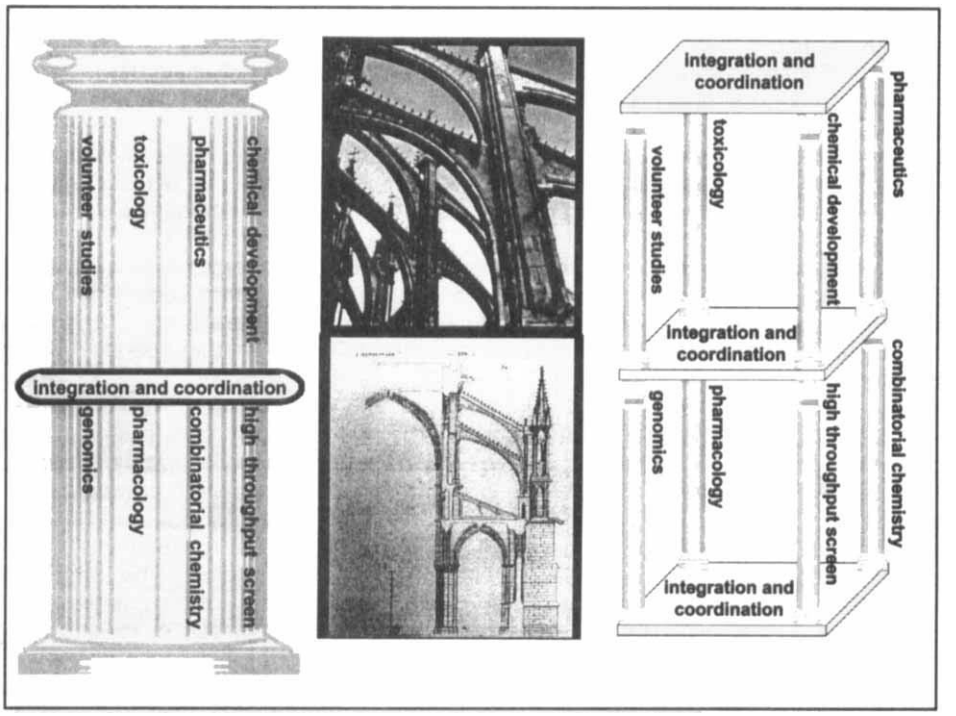

Figure 5 Classical, Medieval and Modern architectural analogies for pharmaceutical R & D

One could say that the collaboration of a large company with a technology provider in a research project, or of a CRO to provide a part of the development work, buttresses the in-house efforts of the large company. If so, the down-sizing of the past few years has left a structure within the large company analogous to the central portion of a large cathedral, a structure that is not self-sustaining. Equally, the buttress cannot be left without a large company for support—it too will collapse. The need for, and permanence of mutual dependence is suggested too by recent developments in the biotechnology sector. In 1995, the funding of the biotechnology sector reached $3.6 billion through alliances, over twice that of 1994, and equivalent to around 10% of total pharmaceutical R & D spending. However, the relative weight of the participation by each partner has still to be optimised. In the Middle Ages the correct amount of buttressing that was necessary for structural integrity was established by trial and error, and of course there were failures, leading to a rather empirical approach to cathedral design (16). As partnerships in the industry are becoming a more common approach, how do we optimise the most efficient relative weighting between intra- and extramural resources; is the stability of a large pharmaceutical company required for such partnerships to work properly, or can their co-ordinating role can be supplanted by yet another small company?

There are an increasing number of examples of partnerships between small companies that avoid the multinationals entirely. A pattern is emerging, in which the three technologies of combinatorial chemistry, high throughput screening and genomics are being used as core elements in drug research and discovery programs, and they are being provided by a number of small companies, or technology providers.

In some cases, large companies have taken the opportunity to build consortia of small companies, with their finances and powers of co-ordination driving the program. Rhone Poulenc Rorer (RPR) and Pfizer are key examples of this phenomenon (17).

RPR's venture, known as GenCell represents nearly 20% of their R&D budget and includes around a dozen or so partners. Pfizer's effort is known as PfizerGen has received allocations of about $200 million per year. Again including around a dozen partners, genomics features prominently, with screening automation, combinatorial chemistry, drug delivery and new therapeutic approaches external to Pfizer's existing portfolio. Schering has avowed a similar strategy (18). These companies are not alone: in the proposed merger between Glaxo Wellcome and SB, potential conflicts between over 200 external collaborations would have needed to be managed.

These examples represent a major change in research strategy, wherein industrial and academic components find themselves increasingly rubbing shoulders. The role of the modern multinational pharmaceutical company is quite different from its traditional one, focusing increasingly on co-ordination and integration rather than operating solely with in-house technical expertise.

Returning to the architectural analogy, we have seen profound advances in building design since the Gothic cathedral. In the last century, the first skyscrapers of Chicago were built with the benefits of new materials with an improved strength to weight ratio, and were the first to use a steel framework based on horizontal and vertical steel components, which could then be faced with brick. An architectural model of this type of pharmaceutical R & D is representable by a box-girder structure. The horizontal elements of integration and co-ordination connect the technology providers, represented by the vertical elements, and in architectural terms, they are essential for structural integrity. In the industry, these are figurative representations of the virtual companies. The structure shown has a number of advantages over the monolithic single-site structure of the traditional pharmaceutical industry, and may represent a means by which a network of entirely small companies can effectively perform drug R & D. Although the structure is suggestive of permanency, one needs to realise that as a project proceeds, the components of the framework will change: alliances are often designed to operate for only a portion of the drug development process.

This structure is light for its height; the thin columns of the frame represents specific technologies essential for the project to succeed, provided by single discipline providers. Many of the peripheral elements which are embedded in the single-column, large-company format, and represent the substantial overheads of that format, are either unrepresented, or brought in as needed. It is flexible; the pillars of specific disciplines of research and development are but examples of what may be needed to compose a total project. These may be increased or curtailed depending on the nature of the project at hand, and its temporal stage of development.

The extreme of outsourced pharmaceutical R & D is found in the virtual company, with exemplars of Vanguard Medica in the UK and Triangle in the US. Vanguard has carried through the anti-migraine drug from SmithKline Beecham (VML-251) from phase I to phase III (19). Successful virtual research is rarer though increasingly the new technologies of genomics, high throughput screening and combinatorial chemistry are now easily available by contract through a great range of small company technology providers. Additional types of work are available through other niche technology companies. Napp, in collaboration with its associated companies Mundipharma and Purdue Pharma, successfully brought a new phosphodiesterase 4 (PDE4) inhibitor from conception through to phase I volunteer trials, initially in the absence of any in-house biological investigative resource (20).

Virtual companies have advantages over traditional company structures, particularly in a modern, rapidly changing environment. They can pick and choose from the best practice available; can acquire the most up-to-date technology; they do not need to invest in large amounts of capital equipment; they can form partnerships or contracts in

a flexible fashion; as small companies, they can reach important decisions quickly; and they can disengage more easily from failed projects. This last point is important, since the cost of a failed project is much more than the naked costs of the project itself. Every additional month spent on a project that ultimately fails is a month lost to an opportunity that could be successful. Curtailing in-house projects is notoriously difficult. Even though there are examples of drug research which survived managerial *coups* (notably the first ACE inhibitor captopril) and were ultimately successful, many other projects have proceeded far longer than is necessary, partly because an internal (*ergo* visible) project champion can easily defend their continuance. Outsourced projects are much more difficult to justify in the absence of solid progress or a clear expectation of progress. Objective criteria are far easier to sustain when the costs of maintaining a partnership are clearly visible in the revenue budget as a dispensible item. In the end, this objectivity feeds into reduced costs and improved efficiency. Furthermore, reduced costs enable profitability for niche products.

This last point is important: the cost of discovery and development for the traditional new pharmaceutical makes products with peak sales of less than $350m per year unlikely to be commercially viable. Reducing this cost by 'virtualisation' enables the possibility of entry into hitherto untapped markets. For those that doubt the economic possibilities for the outsourced development project, a company in the US, CollaGenex filed an NDA for a product for periodontitis, having carried out a slightly abbreviated developmental program up to and including NDA filing in 1996 for a total cost of less than $6 million. The work was conducted at a number of well-established CROs (21).

Although the effort of co-ordinating the component parts of a virtual organisation is in itself a significant expense, requiring significant resource allocation, the advantages of 'virtualisation' are apparent in many areas of business, from automotive manufacture to airline operation. It is no surprise that advocacy for its adoption in the field of pharmaceuticals is growing, particularly among the consultancy firms McKinsey and Andersen Consulting (8,22).

The architectural analogy establishes the conceptual difference between the technology provider and the virtual company, whose sole role is to integrate the activities of others. It also clarifies the difference between the technology provider and the multi-disciplinary research project partner, which can be considered as providing a complete stage in the vertical development of the total R & D construct. It can be seen that the purposes of the CRO, the academic research group or the small entrepreneur may be similar, although the ability of each to fit into the framework depends on the size and shape of the column that is required. Most of the virtual companies, particularly those which aim to carry a project through late development, use CROs extensively, if not exclusively for the work they need to conduct. Those that operate in the discovery phase typically employ biotechnology or academic partners. The difficulty of organising this network in a successful manner should not be underestimated. The structure needs to be sufficiently robust to cope with the problems of delay, which can produce consequential slippage through difficulties in rearranging studies at collaborating organisations, or even withdrawal through financial failure. Although these components have been represented as thin planes, in reality sufficient resources have to be placed in these horizontal components to facilitate the complex co-ordination of the project. This point is exemplified by Vanguard Medica, which although being regarded as a virtual company, employs about 40-50 people to co-ordinate the developmental activities of 6 projects.

A key component for the success of the network of providers is communication. Single-site projects operate substantially through decisions made at meetings, where all the disciplines and people involved in the project may be represented. This is not possible with distantly separated groups, where the bulk of communication will be by

telephony, and communications are a much more important element. The improvements in electronic communication over the past 10 years are of crucial importance to the feasibility of collaborative work in general, although the changes in the next 10 are likely to be even more dramatic. Information technology (IT) is a powerful tool with the potential to change fundamentally the way companies carry out research, and has great applicability to devolved projects. The term 'collaboratory' is entering our vocabulary (23) to describe collaborative research in which research findings can be posted to a common notice board and viewed by other parties with granted access rights as soon as they are available. Issues of security are particularly important for the pharmaceutical industry, and the substantial research towards the establishment of secure links via the Internet will be of key importance for future collaborative activities.

Conclusion - Modern pharmaceutical research places much emphasis on the integrative processes, in an effort to achieve more with less. This is not unlike the adaptive changes to structure which accompany the ageing of the human brain. Increasing integration becomes a compensatory mechanism for the decline in numbers of functional nerve cells which affect us all from our 20s onwards. Greater numbers of synapses enable people to maintain their mental faculties by using their remaining resources more effectively. The human race is successful in large measure because of the brain's plasticity in the face of mortality. The pharmaceutical industry is currently demonstrating similar adaptation as it prepares to enter the next millennium.

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Chapter 35. Chemoinformatics: What is it and How does it Impact Drug Discovery.

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Introduction - The use of information technology and management has become a critical part of the drug discovery process. Chemoinformatics is the mixing of those information resources to transform data into information and information into knowledge for the intended purpose of making better decisions faster in the arena of drug lead identification and optimization. See Figure 1. For example, a medicinal chemist wants to start a new project on α1-subtype receptors in the prostate. After a quick sequence alignment of the seven membrane spanning sequence for the receptor, it is determined that there is a critical mutation in the fifth helix of a Ser to an Ala. To exploit this small but significant difference the researcher probes the internal and external database for small molecules that have affinity for the α1 receptors found in the periphery. The scientist finds several hundred examples comprised of seven basic templates. The template that will be used needs to be judged for synthetic feasibility for a chemical library, selectivity and patentability. Once these factors are weighed for each of the seven basic templates, one or two are chosen and a chemical strategy is defined. A few grams of the basic template are ordered from the intermediates scale-up group and 40 diverse monomers are ordered from internal and external sources. The scheduling for the synthesis and testing of these molecules is finished. A list of available molecules similar to those just designed is located in the internal inventory, a dispensing request is made and a ship date given for the fulfillment of the order to the screening facility. Along the above process, many systems are involved which today typically do not work well together. Chemoinformatics is the discipline which will reduce the cycle time for this process from many weeks to a few days.

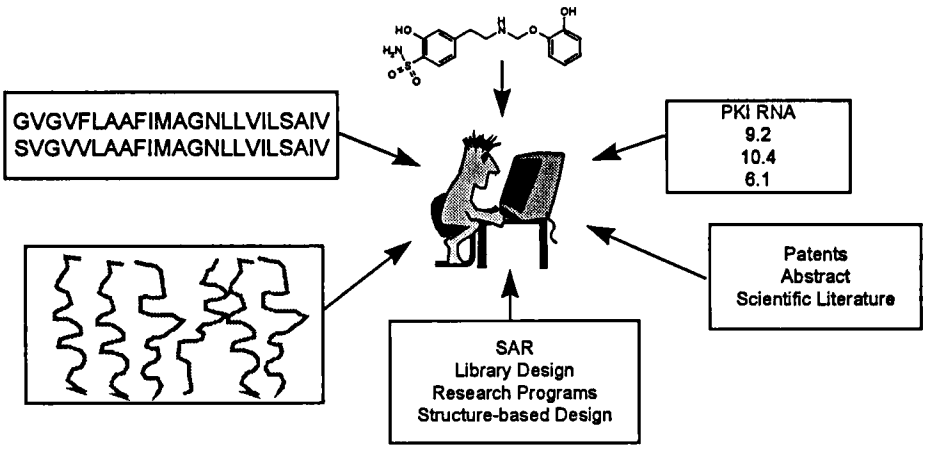

Figure 1. Chemoinformatics brings together all the information resources that a scientist needs to optimize the properties of a ligand to become a drug.

One of the main reasons the activities in the above example do not fit together is that each was designed to optimize one process and the data relationships and structures are not consistent. This inconsistency is the root cause of the interoperability between the processes. One simple way around the data consistency problem, in this very narrow data space, is to create relationships through categorization. Much like a card catalog can be used to help find books by subject, author, etc., this concept can be used to group data and make it easier to find

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and ship around. The best rule when using a catalog is to keep the terms simple and the terms should have an exact scientific meaning. The word "active" often is found in many databases that contain biological results; however, active does not have an exact meaning and changes over time and from research group to research group. Thus it should not be in the database.

To better understand how to weave the information resources together, first the question must be known. Once the questions are known, then the many systems can be retro-designed to make sure the proper data are collected, stored in an easy to retrieve form, and analyzed. The end result of these systems is analysis.

Definitions - As in many disciplines, the vernacular is one of the most difficult barriers to cross in the understanding of that discipline. A few definitions will be given for the terms that will be used for this chapter. It is important to define what Chemoinformatics is; it is equally important to define what it is not. There is another process, Chemometrics, which is the use of computational chemistry methods to drive descriptors, e.g., cLogP; however, this is the optimization of a process and not decision support. Chemometrics is one of the threads in the weave of Chemoinformatics, but not the entire cloth. Information technology is defined as those systems both hardware and software that enable the transactions to take place. For instance, if you were to compute cLogP, the following would happen: the request to compute the cLogP would be set from the desktop computer to the main computational computer and then calculated for the given molecule and finally the result set to the desktop. This is all technology, the computers, the wiring, and even the algorithm to compute the cLogP. Information management is the meaningful use of these technologies to organize data and procedures to answer questions or derive hypotheses. For instance, if you wanted to explore the relationship between cLogP and activity, you would need a database of compounds, sometimes called a registration system, a database of biological results, an algorithm to compute the cLogP, and the hardware systems to compute and transfer the information. The organization of each of the components, the databases, the delivery of the information to the desktop in an efficient manner is information management, etc., while all the individual pieces are technology. The relationship of individuals to a team, is the relationship of information technology to information management. Just as a team is assembled to derive a result greater than its individuals, so are the components of information technology assembled to optimize a process or answer a specific question.

It is also important to define data, results, information, and knowledge. Data are the raw information, like counts from a radio-ligand binding experiment, which are observed. Results are defined as the reduction of those counts to a percent inhibition or several percent inhibitions to give an IC_{50}. Information is the gathering of many results to have a simple understanding. Knowledge is the bringing together of experience and information to generate a rule. If over many examples, it was shown that 2' substituted phenethylamines were always active in α and never in β receptors, this would be knowledge. The entire test in α would be information, the % inhibitions would be results and the raw counts data.

It is also important to define a few processes: collect, reduce, store, report, and analysis. Collect is the process of getting the raw data off the instruments and ready to be reduced. Reduce is the term used to indicate that the raw data have been transformed into a result. Store is the holding place, database or table, for the data to allow access by a single source or multiple sources. Report is the delivery of the information in a concise manner either to the screen or printed on paper. Analysis is the process used to bring forth information and knowledge from the results.

THE QUESTIONS ARE IMPORTANT, SO WHAT ARE THE QUESTIONS?

In Chemoinformatics there are really only two questions: (1) what to test next and (2) what to make next. There are many other questions that are asked, but they can all be judged as to their value to the two primary questions. This chapter will not spend much time on defining

all the questions, but will use a few to illustrate the point of how to define, implement and use information resources to create a Chemoinformatics system. The main processes within drug discovery that will be discussed are lead identification, where a lead is something that has activity in the low micromolar range, and lead optimization, which is the process of transforming a lead into a drug candidate.

The questions asked within the processes are quite different than those between the processes. Within a process, the questions are usually directly toward speeding up the process. For example, within the lead identification process, a biologist will be tasked with screening 100,000 molecules in less than a week for a given screen. To accomplish this, they will want to get all the plates in to the laboratories, know what is in all the samples, understand the protocol, collect all the data, reduce the data to results, post the results to a database, and allow colleagues to create reports on the results. To accomplish this task today is very different from the past. When there were only a few samples to test, this could easily be accomplished manually. The scientist could easily track the results in a lab notebook, record the results on a sheet of paper, and pass it out at a team meeting. The difference today is that the results will fit on several sheets of paper, one could never stay competitive entering the data by hand, or possibility compute all the results on a hand calculator. So, the information group introduces the ability to track samples and compute results in minutes. The information group has just speeded up the process by answering questions as to what is in the well, etc. Making things go faster is valuable, but not as valuable as having the correct information at the time you need it to make a decision.

Making decisions is what happens between the processes. That is, once a handful of leads are verified, a decision as to those to take forward into lead optimization must take place. The decision of which compound to take forward is one that involves many data sources and weighting of importance of each information source. The information sources that are most often employed are: the potency from the high throughput screening, synthetic ease to implement a chemical strategy around a template, the patentability of the molecules arising from the chemical strategy, the selectivity of the leads and this class of compound, ADME (Absorption, Distribution, Metabolism, Excretion) and toxicology information or predictions. While all of these sources of information are important, rarely are they all used in the decision making process. Historically, so few leads were found that only two parameters were important: potency and synthetic ease. This was due to the fact that there was very little profiling of molecules across multiple screens, and since the optimization was a lengthy period, most chemists believed that they could engineer out ADME and toxicology problems. The outcome of this process was a 50% dropout rate in the preclinical evaluation period, and 50% of those that made it through preclinical studies were eliminated in early clinical trials for side effects. Thus, very few drugs came from an enormous amount of work from the Discovery groups.

Discovery groups today cannot afford to have 75% of their efforts wasted. There is now a general awareness that many of those information sources are just as important as potency and synthetic ease. Glaxo-Wellcome realized the importance of these other information sources about 5 years ago. This changed the critical path of a chemical program to include more selectivity analysis, more ADME and toxicology studies in the Discovery process and this has resulted in 24 out of 28 drug candidates to survive the preclinical process 85% survival rate vs. 50% industry average (1). This realization that a modern Discovery group needs all these sources of information to converge at the correct time and in a timely fashion brings about the paradox that Chemoinformatics will solve. The paradox is, even if the Discovery groups have access to all the information, and the systems were built to get information in, not out. Also, information systems were almost always built for a single optimization as given above and not the uniting of the information sources.

Now the research scientists want all these data but cannot get to them even though they are in a database. If they can get to the information and they do not generate the data, they cannot understand it. If they can get the information and understand it, they cannot get it merge

or process it by analysis tools without many file manipulations and transformations. Why is it so difficult? It is so difficult because the information systems were built to optimize a single step within one of the processes, and not designed to optimize decision making. To optimize decision making, one must first determine those generic questions that are at the root of success for the organization, then retro-design the system to answer the questions.

DESIGNING THE SYSTEM

The system design must first start by understanding the data ownership dilemma in a Discovery organization. In most systems the data ownership resides with the database administrator. This is historically so due to the fact that most databases are a part of financial transaction systems, e.g., banking records. Data entry personnel may not care passionately about the data; however, scientists do hold their data personal. This is a major problem because everyone cannot have database rights, but you do want to give complete control over the data to the scientist. Thus, you must have methods to allow the scientist to easily post results to the database and edit results from the database. The scientist should feel comfortable that they have control over the results, not the database systems. The end result should be a sharable database so that all the scientists in the organization can view the best possible results.

The design of the system is dry and uninteresting to most scientists; thus, this section will only outline a few simple principles that make it easier for scientists to get the data they want. The statement that is usually bantered about is "garbage in garbage out". This is a complete over simplification. The results going into the database are have high value however, the information content coming out is low. The information content is low because the results were being put into the database only as a large file cabinet and not as knowledge center. The results are often not linked to a protocol from the experiment, there is no definition to the terms and the input is not peer reviewed for understanding. Thus, the results are only valuable to the person who posted the data and not the entire organization.

There are two simple design schemes that will be discussed. First, the system must be designed backward from the answer to the data that are being captured; second, systems should be in components where each component has one simple task. Designing a decision-making system is like making a molecule. The chemist determines the final product and retro-synthetically designs the molecule. The same is true with the information system design for decision support. If you want to answer the question, how much drift in the experimental result is seen for sf9 cells, then you must make sure that the cell line information is tagged to the results and that you can find all the results over many protocols that use sf9 cells.

The second simple design idea to keep in mind is the idea of modular systems that can plug and play into other systems. One of the major legacies for most information systems is the problem of "silo" systems. The "silo" system is one that is built to collect, store, and report one laboratory's data. This is usually the result of a single person maintaining a database for a discovery team. Each "silo" system holds the data differently and may be in different technology, e.g., some in FileMaker Pro and some in Oracle. Since the data are held in different ways and using different technology, the results in the systems cannot easily be interchanged. This is a very common problem due to the fact that no one was asking or realized the value of looking at all the test results at one time.

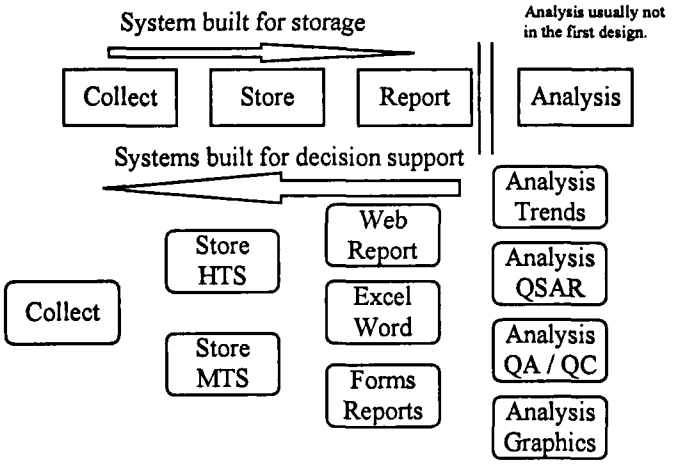

Figure 2. The top diagram defines the way most transactional databases for financial systems are built. Most are built just for keeping the records and simple accounting work with no need for complex analysis. However, scientific data need to be analyzed to develop knowledge from the information. Thus, when designing a scientific information source, start with the question, which will define the analysis methods to be used, which will define which data to collect.

The use of centers of excellence would be one way to remedy the organizational problem that lead to the "silo" systems described above. If you assembled a team to deliver each part of the system, one group for data collection, one for databases, one for interfaces, then all the systems would need to talk to each other. This approach mandates the use of a component architecture-giving rise to a plug and play environment. The major component to success is a common data structure and a common protocol to pass data around.

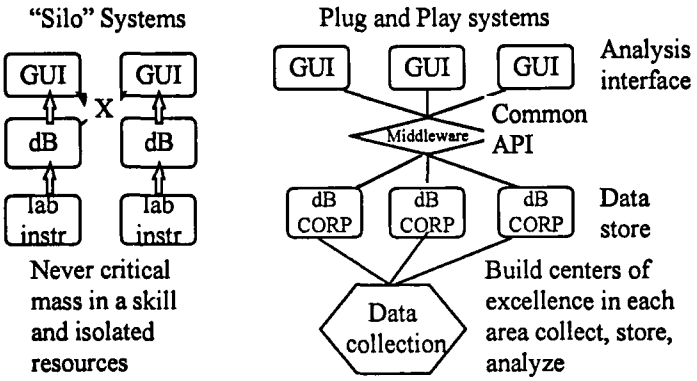

Figure 3. The "silo" system design is the legacy method and the plug and play system is the new design. This is as much a corporate structure and resource problem as it is a technical problem.

BIOLOGICAL DATA SYSTEMS FOR COLLECTION AND STORAGE OF INFORMATION

At this point, the reader should have some conceptual understanding of what it takes to create an adequate information system for the discovery of drug candidates. The next sections will discuss the available software and publications that can be used to build this system from it components. The systems will be discussed as two major parts: systems for organizing biological and chemical information. Using Figure 4 as a guideline for biological information, we will start from collecting information. It takes the assembly of many products and in-house programs to create the systems, because there is no single vendor or system that can offer all the functions described above. Most systems used to collect the raw data are home grown and built around the physical devices that are in the laboratories. Each hardware vendor has a different file format and some use binary and some ASCII. Thus, over the years most companies have gathered their own bag of tricks for solving this problem. However this is time consuming and costly to keep up with. In the last few years, several software vendors have been offering these services, IDBS - Activity Base, OMG - RS^3/HTS, and MDL – Screen (2-4). These products will generally take data from the detector, transform the data into % inhibition, IC_{50}, etc,; then post the data to a database. This would be the "Collect Process" which is in the laboratory. The exact way this is done is not as important as is the fact that these systems should be easy use, quick to get data into and the number crunching needs to be robust (5). The key to success in these systems is ease of use and ease of adaptability. Adaptability is important because it allows the systems to be modified for all types of assays, both *in vitro* and *in vivo*. This is usually the downfall of most commercial systems; that is, they are designed to meet the average needs, and not all the needs. Thus, no solution from a vendor will ever meet all your requirements, but if it can be quickly modified by the in house staff, scientist or programmers, then it is the best possible solution.

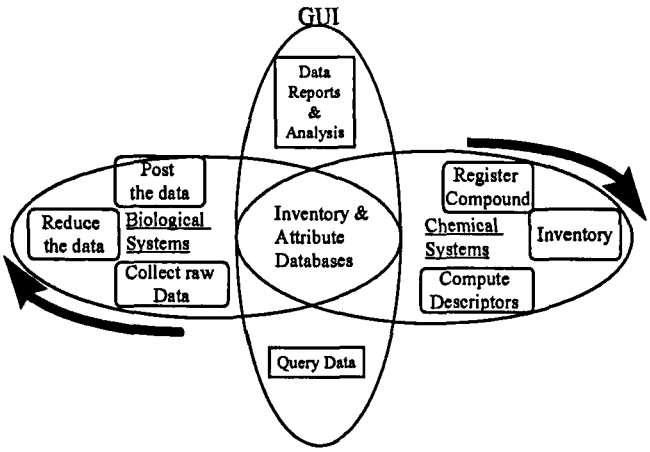

Figure 4. Model of the individual systems and their connections

One of the most common templates for a user interface is to use a table structure. The commonly used table structure is EXCEL, and then macros are used to crunch the data (5). Once the data are accepted as being "good" data, they are released by the scientist to be posted to the "Shared Database". The posting usually happens by highlighting the data in the Excel tables sending it to a relational database via ODBC. The most commonly used database products that are used are Oracle, Sybase, Informix, SQL server, and Access (6). Now the data

are in the database, the main objective will be to make the data easy to understand and easy to get out. The only reason to store the data in a database is to make them easy to find.

The construction of the database for "sharing Information" is based on the ability to browse through the data and retrieve the data. Browsing data in a database is simplified by establishing relationships that group data into concepts and hierarchy. For instance, organizing under therapeutic area, receptor type, etc. The best model to use for organizing the data is the way a library uses a card catalog. The catalog directs you to the information and the book you find has an index. The most important thing is that the catalog and the book index serve quite different functions, the catalog locates and organizes the data and new concepts for organizing the library can be added to the catalog without affecting the location or contents of the book. The index in the book is static and only describes the book. The book represents the table of information that was generated in the above "Collect Process". Once it is loaded into the database it should not be conceptually changed, only more rows of data should be added. New concepts on how to relate this to other tables in the database should be done in related tables, not in the primary data table. This way, as new concepts are deemed important to understanding the data, the only place they need to be added is in the description of the table and not to every row in the database.

A few companies have constructed two separate databases to describe their data. A catalog that knows how to find the data by relationships, what the data fields are, and how to extract the information. The second database is the primary data from the screening process. There can be many of the primary database throughout the company, but there needs to be only one catalog that knows how to locate the information. Just as the card catalog can locate any book without needing to hold the entire content of the book, the database catalog contains only reference to the data, but not any primary data. The RS3 (OMG) (3) and Gemini (CAS) (7) products are the only products in the drug discovery software arena that use this concept, however, their are many similar products for financial systems. The MDL-ISIS product uses the concept but implemented in a hierarchical structure and not in a relational structure (4). The only product that is constructed for browsing both external and internal data is the CAS Gemini product (7).

CHEMICAL DATA SYSTEMS FOR THE COLLECTION AND STORAGE OF

Chemical information systems have been much more standardized over the years and are often called registration systems, with the best known system being MACCS from MDL. However, there are several registration systems, Daylight (8), OMG-RS^3, COUSIN – Upjohn (9), Accord for Access (10) and CAS-Gemini (7). However, this is a very limited view of a chemical system and in the next section many elements from various systems will be highlighted and joined to construct a much broader definition of a chemical information system, but in no way will this be a complete description given the pace of evolution in this type of software. To adequately define a chemical information system such a system must include, registration, computed and measured properties, chemical descriptors, and inventory. The primary purpose for a chemical information system is to be able to identify a chemical substance, find compounds similar to the target compound and determine the location of the compound.

To effectively build the chemical system of the future, an object definition of the chemical sample is paramount. The object design by Purvis, is one of the first object based designs to be published for describing a chemical information (11). The basic concept from the design is that its focus is not just a compound, but rather the sample. Most registration systems concentrate on the compound being equivalent to the sample. This is often true, but in many cases there are several compounds in a sample, or there is no determination of the molecular structure, e.g., natural products. Just like the biological system, the chemical system needs to be easily adaptable. One of the first systems to be developed to implement object technology and build toolkits for extending the functionality of the system was the Daylight software (8) Using the sample object allows for simple change and additions to the description of the sample

without a complete rewrite of the data structures. EMAX solutions uses this sample concept at the core of their inventory system which makes it adaptable from a simple inventory system up to a complex system like haystack (12).

The hub of the chemical information system is the inventory system. The registration system was first used in conjunction with paper or simple inventory systems 15+ years ago. However, as the collection of compounds became greater than a bench top then the need for an electronic record keeping became apparent. An inventory system was added to the registration system. After the inventory systems where added, then computed attributes of the molecule, like MW, cLogP, etc. were included in the database. Soon, there were a half dozen systems tied to the registration system. Registration systems also used registration number as the way the relate the information from on table to another and these registration numbers often had meaning built into them. The registration number should be considered a name and not the primary key for building relationship given the complexity of parsing needed to break the registration number into its pieces.

To correct many of the problems in the old systems, two things need to happen. First, the primary key should be a simple integer and the central database should be the inventory of samples. Samples would then have attributes, for example, the registration number would be treated more like a name and therefore be an attribute and not a key index. Other attributes would include calculated properties (chemometrics) (13), measured properties, container type, location, phase, volume, etc. One of the most difficult attributes to describe and store is a description of the 3D coordinates of all of the conformations. However, a simple integer number without the complexities of a registration number could link all several databases.

THE GRAPHICAL USER INTERFACES

The two most useful GUIs are the Query interface to the database and the Report / Analysis interfaces. The first basic rule it that no one likes to read manuals, so the interface should be self explanatory and each interface should do only a handful of task (14). The most common mistake is to keep adding functionality to an interface rather creating a new interface. This usually results in losing the concept of the first interface and not fully implementing the second interface. So, each interface should do one thing. This then means that the different interfaces need to operate seamlessly. The tight integration is accomplished through a consistent data stream or file structure for chemical information (15).

The most common problem with interfaces is that they all look and operate differently, thus, the scientist wants only one interface. This one interface statement is usually accompanied with the statement that they want everything on the Web. While there are some nice Web tools available, ChemWeb (16) Molecular Spreadsheet (17) and Accord for Web (11) the statement always means a common look and feel to the interface. One of the best ways to get a common look and feel is to mimic those report forms that scientists use to present data in the literature, a table (11). This theme has been used in Accord for Excel, DIVA, Molecular Spreadsheet, Project Leader, and more. There are a few features in each of these that are very useful. For Accord for Excel, the best concept is that it is in a tool that is already familiar to most scientist, DIVA can be completely driven without a manual with a small set of operational buttons, Project Leader footnotes the table with the references to the calculations that where used and Molecular Spreadsheet holds 3 D structures (3,11,17).

The other common interface is a forms based interface, much like the 3x5 index cards of the past. Hagadone and Schultz demonstrated how to build such an interface flexibly in Access and join it to a registration system (18). This is also the method that is most commonly used for ISIS (4). The forms unique position is that it can be tailored to join a large view of the structure and then just the information you want in see along side the structure. The formatting of the data

into boxes allows the scientist to quickly scan the "3x5" card to exact data they want. This is a very chemistry centric view of the data. DIVA is one interface that allows for both the form and table type view from the same interface by allowing the scientist to quickly switch between display modes (3).

The Query tools are just starting to emerge. There have been simple tools, i.e., ISIS, but more complex tools are becoming very popular. The unmet need of querying data is in the area of joining internal and external data. A simple concept to keep in mind for a query interface is the fact that selecting the type of data and the condition to query for should be on one screen. This is the strength for Client based software, at this time, over Web tools. Often with Web tools, many pages or forms will pop up to accomplish a rather complex query, but with a client-based application this can be accomplished without all the extra screens. The other major need in a query tool is the ability to record and replay a query, often called a "canned query". The canned query can be used by anyone with permission from the Web, or the client based application.

The other emerging concept for the query tool is the need for relationships between the data. The most critical need is to have consistent terms used to describe the data. This is often a very difficult task to get scientists across a multi-site company to agree on. The other complication is legacy data, either having the wrong terms or no terms at all. This is exacerbated when the external data are considered. There are libraries of terms available but they are usually behind the times and building translators to match in-house terms is difficult. There are a few collaborations in this area that should be mentioned, many of them with CAS. For example, Daylight and CAS, CAS and Synopsys, and there is a lot of attention on the direction of the Gemini project by CAS.

Conclusion - The world of information is becoming more complex and dense very day. Chemoinformatics will need to evolve with this growth in information sources. In the very near future, Chemoinformatics will need to progress beyond the boundaries of the internal information to include external data and bioinformatics. Chemoinformatics is focused on the central object of a chemical sample. This will need to be one of many objects that are held in the library of objects. In bioinformatics the focus is on sequence, therefore there will need to be tools that can provide gateways between the chemical sample and sequence.

The success of Chemoinformatics will depend on it being built on a solid foundation that will allow for flexibility as the discipline evolves. It is equally important that Chemoinformatics continues to be different from chemometrics and computational chemistry. Perhaps the most critical factor for success will be the teaming of discovery scientist with information technologist to jointly define the direction of Chemoinformatics.

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Chapter 36. Glossary of Terms Used in Medicinal Chemistry (IUPAC Recommendations 1997)

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Introduction - The objective of the glossary is to provide in a single document a consistent terminology and concise definitions of terms covering the various aspects of medicinal chemistry. This was felt necessary with regard to the rapid changes occuring in medicinal chemistry and also by the need to establish international definition standards. Effectively the possibility exists that in different countries certain terms may not have the same meaning, in such a case the creation of an internationally accepted definition is particularly justified.

A Working Party belonging to the IUPAC Section on Medicinal Chemistry has therefore been assembled which prepared the present glossary. Concise but sufficiently explanatory definitions have been formulated for about one hundred commonly employed terms which can be considered of particular interest to the medicinal chemistry community. The glossary has been compiled in part from definitions proposed by the Working Party in part from earlier IUPAC glossaries and in part from well-accepted definitions taken from the literature but which were sometimes published in journals or books that may not be readily accessible.

ALPHABETICAL ENTRIES

The glossary has been compiled in part from definitions proposed by the Working Party and in part from well-accepted definitions taken from the literature. In most cases, definitions given here are for specific areas of medicinal chemistry. Some definitions taken from the Glossary for Chemists of Terms Used in Biotechnology (*Pure Appl. Chem.*, **1992**, *64*, 143-168; © 1992 IUPAC) were also included, eventually in a slightly modified form; they are identified by an asterisk*. Others, which appear in the Glossary on Computational Drug Design (*Pure Appl. Chem.*, **1997**, *69*, 1137-1152) and in Glossary for Chemists of terms used in Toxicology (*Pure Appl. Chem.* **1993**, *65*, 2003-2122), are identified by a double** and a triple*** asterisk respectively.

GLOSSARY OF TERMS USED IN MEDICINAL CHEMISTRY

Active Transport* - Active transport is the carriage of a solute across a biological membrane from low to high concentration that requires the expenditure of (metabolic) energy.

Address-Message Concept - Address-message concept refers to compounds in which part of the molecule is required for binding (address) and part for the biological action (message).

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ADME - Abbreviation for Absorption, Distribution, Metabolism, Excretion. *(See also Pharmacokinetics; Drug disposition).*

Affinity - Affinity is the tendency of a molecule to associate with another. The affinity of a drug is its ability to bind to its biological target (receptor, enzyme, transport system, etc.) For pharmacological receptors it can be thought of as the frequency with which the drug, when brought into the proximity of a receptor by diffusion, will reside at a position of minimum free energy within the force field of that receptor.
For an agonist (or for an antagonist) the numerical representation of affinity is the reciprocal of the equilibrium dissociation constant of the ligand-receptor complex denoted K_A, calculated as the rate constant for offset (k_{-1}) divided by the rate constant for onset (k_1).

Agonist*** - An agonist is an endogenous substance or a drug that can interact with a receptor and initiate a physiological or a pharmacological response characteristic of that receptor (contraction, relaxation, secretion, enzyme activation, etc.).

Allosteric Binding Sites - Allosteric binding sites are contained in many enzymes and receptors. As a consequence of the binding to Allosteric binding sites, the interaction with the normal ligand may be either enhanced or reduced.

Allosteric Enzyme* - An allosteric enzyme is an enzyme that contains a region to which small, regulatory molecules ("effectors") may bind in addition to and separate from the substrate binding site and thereby affect the catalytic activity.
On binding the effector, the catalytic activity of the enzyme towards the substrate may be enhanced, in which case the effector is an activator, or reduced, in which case it is a de-activator or inhibitor.

Allosteric Regulation - Allosteric regulation is the regulation of the activity of allosteric enzymes. *(See also Allosteric binding sites; Allosteric enzymes).*

Analog - An analog is a drug whose structure is related to that of another drug but whose chemical and biological properties may be quite different. *(See also Congener).*

Antagonist*** - An antagonist is a drug or a compound that opposes the physiological effects of another. At the receptor level, it is a chemical entity that opposes the receptor-associated responses normally induced by another bioactive agent.

Antimetabolite*** - An antimetabolite is a structural analog of an intermediate (substrate or coenzyme) in a physiologically occurring metabolic pathway that acts by replacing the natural substrate thus blocking or diverting the biosynthesis of physiologically important substances.

Antisense Molecule - An antisense molecule is an oligonucleotide or analog thereof that is complementary to a segment of RNA (ribonucleic acid) or DNA (deoxyribonucleic acid) and that binds to it and inhibits its normal function.

Autacoid - An autacoid is a biological substance secreted by various cells whose physiological activity is restricted to the vicinity of its release; it is often referred to as local hormone.

Autoreceptor - An autoreceptor, present at a nerve ending, is a receptor that regulates, via positive or negative feedback processes, the synthesis and/or release of its own physiological ligand. *(See also Heteroreceptor).*

Bioassay*** - A bioassay is a procedure for determining the concentration, purity, and/or biological activity of a substance (e.g., vitamin, hormone, plant growth factor, antibiotic, enzyme) by measuring its effect on an organism, tissue, cell, enzyme or receptor preparation compared to a standard preparation.

Bioisostere - A bioisostere is a compound resulting from the exchange of an atom or of a group of atoms with another, broadly similar, atom or group of atoms. The objective of a bioisosteric replacement is to create a new compound with similar biological properties to the parent compound. The bioisosteric replacement may be physicochemically or topologically based. *(See also Isostere).*

Bioprecursor Prodrug - A bioprecursor prodrug is a prodrug that does not imply the linkage to a carrier group, but results from a molecular modification of the active principle itself. This modification generates a new compound, able to be transformed metabolically or chemically, the resulting compound being the active principle.

Biotransformation - Biotransformation is the chemical conversion of substances by living organisms or enzyme preparations.

CADD - See Computer-assisted drug design.

Carrier-Linked Prodrug (Carrier Prodrug) - A carrier-linked prodrug is a prodrug that contains a temporary linkage of a given active substance with a transient carrier group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic cleavage.

Cascade Prodrug - A cascade prodrug is a prodrug for which the cleavage of the carrier group becomes effective only after unmasking an activating group.

Catabolism*** - Catabolism consists of reactions involving endogenous organic substrates to provide chemically available energy (e.g., ATP) and/or to generate metabolic intermediates used in subsequent anabolic reactions.

Catabolite - A catabolite is a naturally occurring metabolite.

Clone* - A clone is a population of genetically identical cells produced from a common ancestor. Sometimes, "clone" is also used for a number of recombinant DNA (deoxyribonucleic acid) molecules all carrying the same inserted sequence.

Codon* - A codon is the sequence of three consecutive nucleotides that occurs in mRNA which directs the incorporation of a specific amino acid into a protein or represents the starting or termination signals of protein synthesis.

Coenzyme - A coenzyme is a dissociable, low-molecular weight, non-proteinaceous organic compound (often nucleotide) participating in enzymatic reactions as acceptor or donor of chemical groups or electrons.

Combinatorial Synthesis - Combinatorial synthesis is a process to prepare large sets of organic compounds by combining sets of building blocks.

Combinatorial Library - A combinatorial library is a set of compounds prepared by combinatorial synthesis.

CoMFA - See Comparative Molecular Field Analysis

Comparative Molecular Field Analysis (CoMFA)** - Comparative molecular field analysis (CoMFA) is a 3D-QSAR method that uses statistical correlation techniques for the analysis of the quantitative relationship between the biological activity of a set of compounds with a specified alignment, and their three-dimensional electronic and steric properties. Other properties such as hydrophobicity and hydrogen bonding can also be incorporated into the analysis. *(See also Three-dimensional Quantitative Structure-Activity Relationship [3D-QSAR]).*

Computational Chemistry** - Computational chemistry is a discipline using mathematical methods for the calculation of molecular properties or for the simulation of molecular behaviour.

Computer-Assisted Drug Design (CADD)** - Computer-assisted drug design involves all computer-assisted techniques used to discover, design and optimize biologically active compounds with a putative use as drugs.

Congener*** - A congener is a substance literally *con-* (with) *generated* or synthesized by essentially the same synthetic chemical reactions and the same procedures. Analogs are substances that are analogous in some respect to the prototype agent in chemical structure.

Clearly congeners may be analogs or vice versa but not necessarily. The term congener, while most often a synonym for homologue, has become somewhat more diffuse in meaning so that the terms congener and analog are frequently used interchangeably in the literature.

Cooperativity - Cooperativity is the interaction process by which binding of a ligand to one site on a macromolecule (enzyme, receptor, etc.) influences binding at a second site, e.g. between the substrate binding sites of an allosteric enzyme. Cooperative enzymes typically display a sigmoid (S-shaped) plot of the reaction rate against substrate concentration. *(See also Allosteric binding sites).*

3D-QSAR - See Three-dimensional Quantitative Structure-Activity Relationship

De novo design** - *De novo* design is the design of bioactive compounds by incremental construction of a ligand model within a model of the receptor or enzyme active site, the structure of which is known from X-ray or nuclear magnetic resonance (NMR) data.

Disposition - See Drug disposition

Distomer - A distomer is the enantiomer of a chiral compound that is the less potent for a particular action. This definition does not excude the possibility of other effect or side effect of the distomer *(See also Eutomer).*

Docking Studies - Docking studies are molecular modeling studies aiming at finding a proper fit between a ligand and its binding site.

Double-Blind Study - A *double-blind study* is a clinical study of potential and marketed drugs, where neither the investigators nor the subjects know which subjects will be treated with the active principle and which ones will receive a placebo.

Double Prodrug (or Pro-Prodrug) - A double prodrug is a biologically inactive molecule which is transformed *in vivo* in two steps (enzymatically and/or chemically) to the active species.

Drug*** - A drug is any substance presented for treating, curing or preventing disease in human beings or in animals. A drug may also be used for making a medical diagnosis or for restoring, correcting, or modifying physiological functions (e.g., the contraceptive pill).

Drug Disposition - Drug disposition refers to all processes involved in the absorption, distribution metabolism and excretion of drugs in a living organism.

Drug Latentiation - Drug latentiation is the chemical modification of a biologically active compound to form a new compound, which *in vivo* will liberate the parent compound. Drug latentiation is synonymous with prodrug design.

Drug Targeting - Drug targeting is a strategy aiming at the delivery of a compound to a particular tissue of the body.

Dual Action Drug - A dual action drug is a compound which combines two desired different pharmacological actions at a similarly efficacious dose.

Efficacy - Efficacy describes the relative intensity with which agonists vary in the response they produce even when they occupy the same number of receptors and with the same affinity. Efficacy *is not* synonymous to Intrinsic activity.

Efficacy is the property that enables drugs to produce responses. It is convenient to differentiate the properties of drugs into two groups, those which cause them to associate with the receptors (affinity) and those that produce stimulus (Efficacy). This term is often used to characterize the level of maximal responses induced by agonists. In fact, not all agonists of a receptor are capable of inducing identical levels of maximal responses. Maximal response depends on the efficiency of receptor coupling, i.e., from the cascade of events, which, from the binding of the drug to the receptor, leads to the observed biological effect.

Elimination - Elimination is the process achieving the reduction of of the concentration of a xenobiotic including its metabolism.

Enzyme* - An enzyme is a macromolecule, usually a protein, that functions as a (bio) catalyst by increasing the reaction rate.

In general, an enzyme catalyzes only one reaction type (reaction selectivity) and operates on only one type of substrate (substrate selectivity). Substrate molecules

are transformed at the same site (regioselectivity) and only one or preferentially one of chiral a substrate or of a racemate is transformed (enantioselectivity[special form of stereoselectivity]).

Enzyme Induction* - Enzyme induction is the process whereby an (inducible) enzyme is synthesized in response to a specific inducer molecule. The inducer molecule (often a substrate that needs the catalytic activity of the inducible enzyme for its metabolism) combines with a repressor and thereby prevents the blocking of an operator by the repressor leading to the translation of the gene for the enzyme.

Enzyme Repression* - Enzyme repression is the mode by which the synthesis of an enzyme is prevented by repressor molecules.

In many cases, the end product of a synthesis chain (e.g., an amino acid) acts as a feed-back corepressor by combining with an intracellular aporepressor protein, so that this complex is able to block the function of an operator. As a result, the whole operation is prevented from being transcribed into mRNA, and the expression of all enzymes necessary for the synthesis of the end product enzyme is abolished.

Eudismic Ratio
Eudismic ratio is the potency of the eutomer relative to that of the distomer.

Eutomer - The Eutomer is the enantiomer of a chiral compound that is the more potent for a particular action *(See also Distomer).*

Genome* - A genome is the complete set of chromosomal and extrachromosomal genes of an organism, a cell, an organelle or a virus; the complete DNA (deoxyribonucleic acid) component of an organism.

Hansch Analysis** - Hansch analysis is the investigation of the quantitative relationship between the biological activity of a series of compounds and their physicochemical substituent or global parameters representing hydrophobic, electronic, steric and other effects using multiple regression correlation methodology.

Hapten*** - A hapten is a low molecular weight molecule that contains an antigenic determinant but which is not itself antigenic unless combined with an antigenic carrier.

Hard Drug - A hard drug is a nonmetabolizable compound, characterized either by high lipid solubility and accumulation in adipose tissues and organelles, or by high water solubility.

In the lay press the term "Hard Drug" refers to a powerful drug of abuse such as cocaine or heroin.

Heteroreceptor - A heteroreceptor is a receptor regulating the synthesis and/or the release of mediators other than its own ligand *(See also Autoreceptor).*

Homologue - The term homologue is used to describe a compound belonging to a series of compounds differing from each other by a repeating unit, such as a methylene group, a peptide residue, etc.

Hormone*** - A hormone is a substance produced by endocrine glands, released in very low concentration into the bloodstream, and which exerts regulatory effects on specific organs or tissues distant from the site of secretion.

Hydrophilicity** - Hydrophilicity is the tendency of a molecule to be solvated by water.

Hydrophobicity** - Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non polar molecules. *(See also Lipophilicity).*

IND - Abbreviation for Investigational New Drug.

Intrinsic activity - Intrinsic activity is the maximal stimulatory response induced by a compound in relation to that of a given reference compound *(See also Partial agonist)*

This term has evolved with common usage. It was introduced by Ariëns as a proportionality factor between tissue response and receptor occupancy. The numerical value of intrinsic activity (alpha) could range from unity (for full agonists, i.e., agonist inducing the tissue maximal response) to zero (for antagonists), the fractional values within this range denoting partial agonists. Ariëns' original definition equates the molecular nature of alpha to maximal response only when response is a linear function of receptor occupancy. This function has been verified. Thus, intrinsic activity, which is a drug and tissue parameter, cannot be used as a characteristic drug parameter for classification of drugs or drug receptors. For this purpose, a proportionality factor derived by null methods, namely, relative efficacy, should be used. Finally, "intrinsic activity" should not be used instead of "intrinsic efficacy". A "partial agonist" should be termed "agonist with intermediate intrinsic efficacy" in a given tissue.

Inverse Agonist - An inverse agonist is a drug which acts at the same receptor as that of an agonist, yet produces an opposite effect. Also called negative antagonists.

Isosteres - Isosteres are molecules or ions of similar size containing the same number of atoms and valence electrons, e.g., O^{2-}, F^-, Ne *(See also Bioisostere).*

Latentiated drug - See Drug Latentiation.

Lead Discovery - Lead Discovery is the process of identifying active new chemical entities, which by subsequent modification may be transformed into a clinically useful drug.

Lead Generation - Lead Generation is the term applied to strategies developed to identify compounds which possess a desired but non-optimized biological activity.

Lead Optimization - Lead optimization is the synthetic modification of a biologically active compound, to fulfill all stereoelectronic, physicochemical, pharmacokinetic and toxicologic required for clinical usefulness.

Lipophilicity** - Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behaviour in a

biphasic system, either liquid-liquid (e.g., partition coefficient in octan-1-ol/water) or solid/liquid (retention on reversed-phase high performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system). *(See also Hydrophobicity).*

Medicinal Chemistry - Medicinal chemistry is a chemistry-based discipline, also involving aspects of biological, medical and pharmaceutical sciences. It is concerned with the invention, discovery, design, identification and preparation of biologically active compounds, the study of their metabolism, the interpretation of their mode of action at the molecular level and the construction of structure-activity relationships.

Metabolism* - The term metabolism comprises the entire physical and chemical processes involved in the maintenance and reproduction of life in which nutrients are broken down to generate energy and to give simpler molecules (catabolism) which by themselves may be used to form more complex molecules (anabolism).

In case of heterotrophic organisms, the energy evolving from catabolic processes is made available for use by the organism.

In medicinal chemistry the term metabolism refers to the biotransformation of xenobiotics and particularly drugs. *(See also Biotransformation; Xenobiotic).*

Metabolite - A metabolite is any intermediate or product resulting from metabolism.

Me-Too Drug - A me-too drug is a compound that is structurally very similar to already known drugs, with only minor pharmacological differences.

Molecular Graphics** - Molecular graphics is the visualization and manipulation of three-dimensional representations of molecules on a graphical display device.

Molecular Modeling** - Molecular modeling is a technique for the investigation of molecular structures and properties using computational chemistry and graphical visualization techniques in order to provide a plausible three-dimensional representation under a given set of circumstances.

Mutagen*** - A mutagen is an agent that causes a permanent heritable change (i.e., a mutation) into the DNA (deoxyribonucleic acid) of an organism.

Mutual Prodrug - A mutual prodrug is the association in a unique molecule of two, usually synergistic, drugs attached to each other, one drug being the carrier for the other and vice versa.

NCE - See New Chemical Entity.

NDA - Abbreviation for New Drug Application.

New Chemical Entity - A new chemical entity (NCE) is a compound not previously described in the literature.

Non-Classical Isostere - Same meaning as Bioisostere.

Nucleic Acid* - A nucleic acid is a macromolecule composed of linear sequences of nucleotides that perform several functions in living cells, e.g., the storage of genetic

information and its transfer from one generation to the next DNA (deoxyribonucleic acid), the expression of this information in protein synthesis (mRNA, tRNA) and may act as functional components of subcellular units such as ribosomes (rRNA).

RNA (ribonucleic acid) contains D-ribose, DNA contains 2-deoxy-D-ribose as the sugar component.

Nucleoside* - A nucleoside is a compound in which a purine or pyrimidine base is bound via a N-atom to C-1 replacing the hydroxy group of either 2-deoxy-D-ribose or of D-ribose, but without any phosphate groups. *(See also nucleotide).*

The common nucleosides in biological systems are adenosine, guanosine, cytidine, and uridine (which contain ribose) and deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine (which contain deoxyribose).

Nucleotide - A nucleotide is a nucleoside in which the primary hydroxy group of either 2-deoxy-D-ribose or of D-ribose is esterified by orthophosphoric acid. *(See also nucleoside).*

Oligonucleotide - An oligonucleotide is an oligomer resulting from a linear sequences of nucleotides.

Oncogene*** - An oncogene is a normal cellular gene which, when inappropriately expressed or mutated, can transform eukaryotic cells into tumour cells.

Orphan Drug - An orphan drug is a drug for the treatment of a rare disease for which reasonable recovery of the sponsoring firm's research and development expenditure is not expected within a reasonable time. The term is also used to describe substances intended for such uses.

Partial Agonist - A partial agonist is an agonist which is unable to induce maximal activation of a receptor population, regardless of the amount of drug applied *(See also Intrinsic activity).*

Pattern Recognition** - Pattern recognition is the identification of patterns in large data sets using appropriate mathematical methodologies.

Peptidomimetic - A peptidomimetic is a compound containing non-peptidic structural elements that is capable of mimicking or antagonizing the biological action(s) of a natural parent peptide. A peptidomimetic does no longer have classical peptide characteristics such as enzymatically scissille peptidic bonds. *(See also peptoids).*

Peptoid - A peptoid is a peptidomimetic that results from the oligomeric assembly of N-substituted glycines.

Pfeiffer's Rule - Pfeiffer's rule states that in a series of chiral compounds the eudismic ratio increases with increasing potency of the eutomer.

Pharmacokinetics*** - Pharmacokinetics refers to the study of absorption, distribution, metabolism and excretion (ADME) of bioactive compounds in a higher organism. *(See also Drug disposition).*

Pharmacophore (Pharmacophoric Pattern) - A pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response.

A pharmacophore does not represent a real molecule or a real association of functional groups, but a purely abstract concept that accounts for the common molecular interaction capacities of a group of compounds towards their target structure. The pharmacophore can be considered as the largest common denominator shared by a set of active molecules. This definition discards a misuse often found in the medicinal chemistry literature which consists of naming as pharmacophores simple chemical functionalities such as guanidines, sulfonamides or dihydroimidazoles (formerly imidazolines), or typical structural skeletons such as flavones, phenothiazines, prostaglandins or steroids.

Pharmacophoric Descriptors - Pharmacophoric descriptors are used to define a pharmacophore, including H-bonding, hydrophobic and electrostatic interaction sites, defined by atoms, ring centers and virtual points.

Placebo - A placebo is an inert substance or dosage form which is identical in appearance, flavor and odour to the active substance or dosage form. It is used as a negative control in a bioassay or in a clinical study.

Potency*** - Potency is the dose of drug required to produce a specific effect of given intensity as compared to a standard reference.

Potency is a comparative rather than an absolute expression of drug activity. Drug potency depends on both affinity and efficacy. Thus, two agonists can be equipotent, but have different intrinsic efficacies with compensating differences in affinity.

Prodrug - A prodrug is any compound that undergoes biotransformation before exhibiting its pharmacological effects. Prodrugs can thus be viewed as drugs containing specialized non-toxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule . *(See also Double prodrug).*

QSAR - See Quantitative Structure-Activity Relationships

Quantitative Structure-Activity Relationships (QSAR)** - Quantitative structure-activity relationships are mathematical relationships linking chemical structure and pharmacological activity in a quantitative manner for a series of compounds. Methods which can be used in QSAR include various regression and pattern recognition techniques.

Receptor* - A receptor is a molecule or a polymeric structure in or on a cell that specifically recognizes and binds a compound acting as a molecular messenger (neurotransmitter, hormone, lymphokine, lectin, drug, etc.).

Receptor Mapping** - Receptor mapping is the technique used to describe the geometric and/or electronic features of a binding site when insufficient structural data for this receptor or enzyme are available. Generally the active site cavity is defined by comparing the superposition of active to that of inactive molecules.

Second Messenger - A second messenger is an intracellular metabolite or ion increasing or decreasing as a response to the stimulation of receptors by agonists, considered as the "first messenger". This generic term usually does not prejudge the rank order of intracellular biochemical events.

Site-Specific Delivery - Site-specific delivery is an approach to target a drug to a specific tissue, using prodrugs or antibody recognition systems.

Soft Drug - A soft drug is a compound that is degraded *in vivo* to predictable non-toxic and inactive metabolites, after having achieved its therapeutic role.

SPC - See Structure-property correlations

Structure-activity relationship (SAR) - Structure-activity relationship is the relationship between chemical structure and pharmacological activity for a series of compounds.

Structure-Based Design** - Structure-based design is a drug design strategy based on the 3D structure of the target obtained by X-ray or NMR.

Structure-Property Correlations (SPC)** - Structure-property correlations refers to all statistical mathematical methods used to correlate any structural property to any other property (intrinsic, chemical or biological), using statistical regression and pattern recognition techniques.

Systemic*** - Systemic means relating to or affecting the whole body.

Teratogen*** - A teratogen is a substance that produces a malformation in a foetus.

Three-Dimensional Quantitative Structure-Activity Relationship (3D-QSAR) - A three-dimensional quantitative structure-activity relationship is the analysis of the quantitative relationship between the biological activity of a set of compounds and their spatial properties using statistical methods.

Topliss Tree** - A Topliss tree is an operational scheme for analog design.

Transition-State Analog - A transition-state analog is a compound that mimics the transition state of a substrate bound to an enzyme.

Xenobiotic*** - A xenobiotic is a compound foreign to an organism (xenos [greek] = foreign).

Chapter 37. Glossary of Terms Used in Computational Drug Design (IUPAC Recommendations 1997)

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Introduction - Computational drug design is a rapidly growing field which is now a very important component in the discipline of medicinal chemistry. At the same time many medicinal chemists lack significant formal training in this field and may not have a clear understanding of some of the terminology used but need to grasp concepts, follow research results, define problems for, and utilize findings of, computational drug design.

In this context the IUPAC Medicinal Chemistry Section Committee felt it would be useful to develop a glossary of terms in computational drug design for easy reference purposes. Also there is the possibility that in different countries certain terms may not have the same meaning and in such a case there would be value in trying to establish an international definition standard. Accordingly a Working Party of seven experts in the field was assembled who constructed a glossary of some 100 terms. Concise but sufficiently explanatory definitions have been formulated based on a variety of literature sources and selected key references provided.

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ALPHABETICAL ENTRIES

Some of the definitions also appear in the Glossary of Terms used in Medicinal Chemistry (IUPAC recommendations 1997; *© 1997 IUPAC*). These are marked with an asterisk.

For some definitions the more extended form taken from the Glossary of Terms in Theoretical Organic Chemistry is included in smaller font.

GLOSSARY OF TERMS USED IN COMPUTATIONAL DRUG DESIGN

Ab initio Calculations - *Ab initio* calculations are quantum chemical calculations using exact equations with no approximations which involve the whole electronic population of the molecule.

> *Ab initio* quantum mechanical methods (Synonymous with non-empirical quantum mechanical methods) - Methods of quantum mechanical calculations independent of any experiment other than the determination of fundamental constants. The methods are based on the use of the full Schrödinger equation to treat all the electrons of a chemical system. In practice, approximations are necessary to restrict the complexity of the electronic wavefunction and to make its calculation possible.

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AM1 calculations - AM1 calculations are semi-empirical molecular orbital calculations developed at the University of Austin in Texas (*AM1* = Austin Model 1). These calculations involve the valence electrons of the atoms of the molecule. They are a further development of MNDO calculations (1). *(see MNDO calculations)*

AMBER - AMBER is a well-known molecular mechanics program for calculations on proteins and nucleic acids. *(see Molecular mechanics)*

Artificial Neural Networks - Artificial neural networks (*ANN*) are algorithms simulating the functioning of human neurons and may be used for pattern recognition problems, e.g., to establish quantitative structure-activity relationships.

Atomic Orbitals (AO) - Atomic orbitals are mathematical functions (e.g., Gaussian, or Slater functions) used in quantum chemical calculations. A set of atomic orbitals described by a defined function is the basis set of atomic orbitals. *(see Slater-type orbitals)*

> Orbital (Atomic or Molecular) - A wavefunction which depends explicitly on the spatial coordinates of only one electron.

Basis Set - A basis set is a set of mathematical functions used in molecular orbital (*MO*) calculations, e.g., the *6-31G** basis set used in *ab initio* calculations. 6-31G* and similar expression refers to the type of mathematical function used. *(see Molecular orbital (MO) calculations)*

> Basis set - A set of basis functions employed for the representation of molecular orbitals. One may distinguish the minimal basis set (includes one basis function for each SCF (*SCF = Self-Consistent Field*) occupied atomic orbital with distinct principal and angular momentum quantum numbers); split valence basis set (includes two or more sizes of basis function for each valence orbital); double *zeta* (DZ) basis set (a split valence basis set that includes exactly twice as many functions as the minimal basis set; extended basis set (the set larger than the double *zeta* basis set); polarized basis set (incorporates basis functions of higher angular quantum number beyond what is required by the atom in its electron ground state; allows orbitals to change not only size, but also shape); basis set with diffuse functions and others.

Chemometrics - Chemometrics is the application of statistics to the analysis of chemical data (from organic, analytical or medicinal chemistry) and design of chemical experiments and simulations.

CLOGP Values - CLOGP values are calculated 1-octanol/water partition coefficients, frequently used in structure-property correlation or quantitative structure-activity relationship (*SPC/QSAR*) studies (2) *(see Structure-property correlations (SPC) and Quantitative structure-activity relationships (QSAR))*

Cluster Analysis - Cluster analysis is the clustering, or grouping, of large data sets (e.g., chemical and/or pharmacological data sets) on the basis of similarity criteria for appropriately scaled variables that represent the data of interest. Similarity criteria (distance based, associative, correlative, probabilistic) among the several clusters facilitate the recognition of patterns and reveal otherwise hidden structures (3-5).

CNDO/2 Calculations - CNDO/2 calculations are semi-empirical molecular orbital (*MO*) calculations using complete neglect of differential overlap. (*see Molecular orbital (MO) calculations*)

Comparative Molecular Field Analysis (CoMFA)* - Comparative Molecular Field Analysis (CoMFA) is a 3D-QSAR method that uses statistical correlation techniques for the analysis of the quantitative relationship between the biological activity of a set of compounds with a specified alignment, and their three-dimensional electronic and steric properties. Other properties, such as hydrophobicity and H-bonding can also be incorporated into the analysis (6,7). *(see 3D-QSAR, Hydrophobicity)*

Computational Chemistry* - Computational chemistry is a discipline using mathematical methods for the calculation of molecular properties or for the simulation of molecular behaviour. It also includes, e.g., synthesis planning, database searching, combinatorial library manipulation (8,9).

Computer-Assisted Drug Design (CADD)* - Computer-assisted drug design involves all computer-assisted techniques used to discover, design and optimize biologically active compounds with a putative use as drugs. (*see Drug design*).

Computer-Assisted Molecular Design (CAMD) - Computer-assisted molecular design involves all computer-assisted techniques used to discover, design and optimize compounds with desired structure and properties.

Computer-Assisted Molecular Modeling (CAMM) - Computer-assisted molecular modeling is the investigation of molecular structures and properties using computational chemistry and graphical visualization techniques.

Computer Chemistry - Computer chemistry is often used as equivalent to computational chemistry, and can also refer to the use of computers in synthesis planning (9,10).

Conformational Analysis - Conformational analysis consists of the exploration of energetically favorable spatial arrange-ments (shapes) of a molecule (conformations) using molecular mechanics, molecular dynamics, quantum chemical calculations or analysis of experimentally-determined structural data, e.g., NMR or crystal structures. Molecular mechanics and quantum chemical methods are employed to compute conformational energies, whereas systematic and random searches, Monte Carlo, molecular dynamics, and distance geometry are methods (often combined with energy minimization procedures) used to explore the conformational space. *(see Distance geometry, Molecular dynamics, Molecular Mechanics, Monte Carlo technique, Quantum chemical methods)*

Conformationally Flexible Searching (CFS) - Conformationally flexible searching is a three dimensional-structure database search taking into account the flexibility of molecules.

Connolly Surface - The Connolly surface is the envelope traced out by the point of contact of a defined probe (e.g., a sphere) and a molecule of interest where they touch once, plus the Van der Waals surface of the probe where it touches twice or more (the re-entrant surface), It is used to visualize the molecular surface.

Craig Plot - A Craig plot is a plot of two substituent parameters (e.g., Hansch-Fujita π and Hammett σ values) used in analog design.

CSSR - The CSSR (*Crystal Structure Search Retrieval*) file format is one of several used by the Cambridge Crystal Structure Database (*CSD*) to store molecular structures. This format is used in many molecular modeling software packages.

De Novo Design* - *De novo* design is the design of bioactive compounds by the incremental construction of a ligand model within the receptor or enzyme active site, the structure of which is known from X-ray or nuclear magnetic resonance (*NMR*) data.

Discriminant Analysis - Discriminant analysis is a statistical technique to find a set of descriptors which can be used to detect and rationalize separation between activity classes.

Distance Geometry - Distance geometry is a mathematical method used to build three-dimensional (*3D*) molecular models from a set of approximate interatomic distances (e.g., nuclear Overhauser effect (*NOE*) experiments in nuclear magnetic resonance (*NMR*) suggest only ranges of distances). Distance geometry can be used to define a 3D pharmacophore starting from a set of molecules with the same mechanism of action, or for the generation of likely geometries for drug-receptor complexes using intermolecular distance constraints (11).

Docking Studies - Docking studies are computational techniques for the exploration of the possible binding modes of a substrate to a given receptor, enzyme or other binding site.

D-optimal Design - D-optimal design is an experimental design technique based on the optimization of the determinant calculated from the variance-covariance matrix of the descriptors. It is used to maximize the efficiency of fractional (uncomplete) factorial design. *(see Factorial design, Fractional factorial design)*

3D-QSAR (Three-Dimensional Quantitative Structure-Activity Relationships)* - Three-dimensional quantitative structure-activity relationships (*3D-QSAR*) involves the analysis of the quantitative relationship between the biological activity of a set of compounds and their three-dimensional properties using statistical correlation methods.

Drug Design - Drug design includes not only ligand design, but also pharmacokinetics and toxicity, which are mostly beyond the possibilities of structure- and/or computer-aided design. Nevertheless, appropriate chemometric tools, including experimental design and multivariate statistics, can be of value in the planning and evaluation of pharmacokinetic and toxicological experiments and results. Drug design is most often used instead of the correct term "Ligand Design".

Electrostatic Field and Potential - The electrostatic field and potential are properties of a molecule arising from the interaction between a charged probe, such as a positive unit point charge reflecting a proton, and a target molecule. These fields and potential are being used in three-dimensional quantitative structure-activity relationship (*3D-QSAR)* studies and to compare or assess the similarity of a set of molecules.

> Electrostatic potential - A physical property equal in magnitude to the electrostatic energy between the static charge distribution, r(r), of an atomic or molecular system and a positive unit point charge located at r. The electrostatic potential V(r) that is produced at any point r by the electrons and nuclei (A) of the system is given by i.e. $V(r) = \Sigma Z_A/|R_A-r| - \int r(r')dr'/|r'-r|$.

Energy Minimization - Energy minimization is a mathematical procedure to locate the stable conformations of a molecule (energy minima), as determined by

molecular mechanics or quantum mechanical calculations. *(see Molecular mechanics, Quantum chemical calculations)*

Experimental Design - Experimental design is the use of mathematical and statistical methods to select the minimum number of experiments or compounds for optimal coverage of descriptor or variable space.

Extended Hückel (EH) Calculations - Extended Hückel calculations are low-level semi-empirical molecular orbital (*MO*) calculations.

> Extended Hückel method - A semi-empirical all-valence electron quantum mechanical method which uses the same approximations, apart from π-approximation and neglect of overlap integrals, as those of Hückel molecular orbital theory. The method reproduces relatively well the shapes and the order of energy levels of molecular orbitals. The account for overlap makes it possible to describe the net destabilization caused by interaction of two double occupied orbitals.

Extrathermodynamic Approach - The extrathermodynamic approach involves the correlation between variables which, from a strictly thermodynamic standpoint, are not related. It is the basis of Hansch analysis used in traditional QSAR (12)

Factorial Design (FD) - Factorial design is an experimental design technique in which each variable (factor or descriptor) is investigated at fixed levels. In a two-level *FD*, each variable can take two values, e.g., high and low lipophilicity.

File Format - The (molecular) file format describes the layout of a computer data file. It is a set of instructions on how a molecule is encoded with respect to its connectivity, atom types, coordinates, and may also contain bibliographic data.

Force Field - The force field is a set of functions and parametrization used in molecular mechanics calculations.

> Force field - Within the molecular mechanics approach, a set of potential functions defining bond stretch, bond angle (both valence and dihedral) distortion energy of a molecule as compared with its nonstrained conformation (that characterized by standard values of bond lengths and angles). A set of transferable empirical force constants is preassigned and the harmonic approximation is usually employed. Some force fields may contain terms for interactions between non-bonded atoms, electrostatic, hydrogen bond and other structural effects as well as account for anharmonicity effects.
> In vibrational spectroscopy, the inverse problem is solved of determining a set of force constants and other parameters of a chosen potential energy functions which would match with experimentally observed vibrational frequencies of a given series of congeneric molecules.

Fractional Factorial Design (FFD) - Fractional factorial design is an experimental design technique, using a reduction factor in order to limit the number of experiments to a lower number than obtained by factorial design.

Free Energy Perturbation Calculations - Free energy perturbation calculations are mathematical procedures used in molecular dynamics studies to gradually convert one chemical species to another in a thermodynamic cycle.

Free-Wilson (FW) Analysis - Free-Wilson analysis is a regression technique using the presence or absence of substituents or groups as the only molecular descriptors in correlations with biological activity (12).

Gaussian-type Orbitals (GTO) - Gaussian-type orbitals are mathematical functions used in *ab initio* calculations. They have superceded Slater-type orbitals because of the greater computational efficiency that results. *(see Slater-type orbitals)*

Genetic Algorithm - A genetic algorithm is an optimization algorithm based on the mechanisms of Darwinian evolution which uses random mutation, crossover and selection procedures to breed better models or solutions from an originally random starting population or sample (13).

GOLPE - Generating optimal linear *PLS* estimations. It is an advanced variable selection technique in partial least squares (*PLS*) used in three-dimensional quantitative structure-activity relationships (*3D QSAR*) studies to handle very large data sets. *(see Partial least squares (PLS))*

GRID - GRID is a program for receptor/ligand mapping. It calculates interaction energies between probes and target molecules at interaction points on a 3D grid (14).

Hamiltonian - The Hamiltonian is a mathematical operator function used in molecular orbital calculations (1).

Hammett Constant σ - The Hammett constant is an electronic substituent descriptor reflecting the electron-donating or -accepting properties of a substituent (15).

Hansch Analysis* - Hansch analysis is the investigation of the quantitative relationship between the biological activity of a series of compounds and their physicochemical substituent or global parameters representing hydrophobic, electronic, steric and other effects using multiple regression correlation methodology (12,16).

Hansch-Fujita π Constant - The Hansch-Fujita π constant describes the contribution of a substituent to the lipophilicity of a compound (16).

Highest Occupied Molecular Orbital (HOMO) Energy - The highest occupied molecular orbital (HOMO) energy is obtained by molecular orbital calculations and relates to the ionization potential of a molecule and its reactivity as a nucleophile. (see *Lowest unoccupied molecular orbital (LUMO) energy)*

> Frontier orbital - The molecular orbitals that involve the highest occupied molecular orbital (*HOMO*) and the lowest unoccupied molecular orbital (*LUMO*) of a given molecular entity. In the case of an odd-electron molecular entity, when its HOMO is occupied by a single electron such a molecular orbital is termed a singly occupied molecular orbital (*SOMO*). Depending on the properties of the reactive partner, the SOMO of a given species may function as either HOMO or LUMO. The special importance of the frontier orbitals is due to the fact that a broad variety of chemical reactions takes place at a position and in a direction where the overlap of HOMO and LUMO of the respective reactants is maximal.

Homology Model - A homology model is a model of a protein, whose three-dimensional structure is unknown, built from, e.g., the X-ray coordinate data of similar proteins or using alignment techniques and homology arguments.

Hydrophilicity* - Hydrophilicity is the tendency of a molecule to be solvated by water.

Hydrophobic Fragmental Constant (f or f') - The hydrophobic fragmental constant of a substituent or molecular fragment represents the lipophilicity contribution of that molecular fragment (17-19).

Hydrophobicity* - Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non-polar molecules (20-22).

Indicator Variable - An indicator variable is a descriptor that can assume only two values indicating the presence (=1) or absence (=0) of a given condition. It is often used to indicate the absence or presence of a substituent or substructure. More broadly, it is a variable which can encode anything that the investigator chooses.

Ligand Design - Ligand design is the design of ligands using structural information about the target to which they should bind, often by attempting to maximize the energy of the interaction. *(see Docking studies)*

Linear Combination of Atomic Orbitals (LCAO) - The linear combination of atomic orbitals (LCAO) is a mathematical method used in quantum chemical calculations. It expresses the approximation of the molecular orbital function as a linear combination of atomic orbitals chosen as the basis functions.

Lipophilicity* - Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behaviour in a biphasic system, either liquid-liquid (e.g. partition coefficient in 1-octanol/water) or solid-liquid (retention on reversed-phase high-performance liquid chromatography (*RP-HPLC*) or thin-layer chromatography (*TLC*) system).

Lowest Unoccupied Molecular Orbital (LUMO) Energy - The lowest unoccupied molecular orbital (LUMO) energy is obtained from molecular orbital calculations and represents the electron affinity of a molecule or its reactivity as an electrophile. (see *Highest occupied molecular orbital (HOMO) energy*)

MINDO/3 Calculations - MINDO/3 (*Modified Intermediate Neglect of Differential Overlap*) calculations are semi-empirical *MO* calculations (23).

MM2 Calculations - MM2 calculations involve molecular mechanical calculations using version 2 of the widely-distributed force field program *MM2* (24).

MNDO Calculations - MNDO calculations are semi-empirical molecular orbital (*MO*) calculations, using a modified neglect of diatomic (differential) overlap approximation.

MOL File Format - The MOL file format is used to encode chemical structures, substructures and conformations as text-based connection tables. It is used by MDL Information Systems Inc. (e.g., in their MACCS or ISIS programs) (25).

Molar Refractivity (MR) - The molar refractivity is the molar volume corrected by the refractive index. It represents size and polarizability of a fragment or molecule.

Molecular Connectivity Index - A molecular connectivity index is a numeric descriptor derived from molecular topology (26).

Molecular Descriptors - Molecular descriptors are terms that characterize a specific aspect of a molecule (27).

Molecular Design - Molecular design is the application of all techniques leading to the discovery of new chemical entities with specific properties required for the intended application.

Molecular Dynamics - Molecular dynamics is a simulation procedure consisting of the computation of the motion of atoms in a molecule or of individual atoms or molecules in solids, liquids and gases, according to Newton's laws of motion. The forces acting on the atoms, required to simulate their motions, are generally calculated using molecular mechanics force fields. *(see Molecular mechanics)*

Molecular Electrostatic Potentials (MEP) - Molecular electrostatic potentials (MEP) are electrostatic properties of a molecule based on the charge density as calculated directly from the molecular wavefunction. The electrostatic potential (scalar with dimensions of energy) is calculated at a point in the vicinity of a molecule. The spatial derivative is the electric force (vector) acting on a unit positive charge at that point caused by the nuclei and the electrons of the molecule (28).

Molecular Graphics* - Molecular graphics is a technique for the visualization and manipulation of molecules on a graphical display device.

Molecular Interaction Potentials (MIP) - Molecular interaction potentials (MIP) are field properties arising from the interaction of a probe (e.g., methyl, proton or water) with a molecule. These are calculated in a space around the molecule.

Molecular Lipophilic Potentials (MLP) - Molecular lipophilic potentials are properties on the Van der Waals or solvent accessible molecular surface or any other point in space (e.g., in a 3D grid for CoMFA studies) calculated from atomic lipophilicity contributions. It can be used for log P calculations, CoMFA and docking studies (29).

Molecular Mechanics - Molecular mechanics is the calculation of molecular conformational geometries and energies using a combination of empirical force fields (30).

> Molecular mechanics - (synonymous with force field method) - Method of calculation of geometrical and energy characteristics of molecular entities on the basis of empirical potential functions (see force field) the form of which is taken from classical mechanics. The method implies transferability of the potential functions within a network of similar molecules. An assumption is made on "natural" bond lengths and angles, deviations from which result in bond and angle strain respectively. Repulsive or attractive Van der Waals and electrostatic forces between nonbonded atoms are also taken into account.

Molecular Modeling* - Molecular modeling is the investigation of molecular structures and properties using computational chemistry and graphical visualization techniques in order to provide a plausible three-dimensional representation under a given set of circumstances.

Molecular Orbital (MO) Calculations - Molecular orbital (MO) calculations are quantum chemical calculations based on the Schrödinger equation, which can be subdivided into semi-empirical and *ab initio* methods. *(see Ab initio calculations)*

> Molecular orbital theory - An approach to molecular quantum mechanics which uses one-electron functions (orbitals) to approximate the full wavefunction.

obtained by X-ray or nuclear magnetic resonance (*NMR*) studies, or from protein homology models.

Structure-Property Correlations (SPC)* - Structure-property correlations (*SPC*) refers to all statistical mathematical methods used to correlate any molecular property (intrinsic, chemical or biological) to any other property, using statistical regression or pattern recognition techniques (37).

Swain-Lupton Parameters (*F* and *R*) - The Swain and Lupton parameters (*F* and *R*) are electronic field and resonance descriptors derived from Hammett constants (18).

Taft Steric Parameter (Es) - The Taft steric parameter is a relative reaction parameter encoding the reaction rate retardation due to the size of a substituent group.

Three-Dimensional Database Searching - Three-dimensional database searching is a lead finding technique using three-dimensional structures of compounds stored in a database.

Topliss Tree* - A Topliss tree is an operational scheme for analog design (38).

Topological Index - A topological index is a numerical value associated with chemical constitution for correlation of chemical structure with various physical properties, chemical reactivity or biological activity. (see *Molecular connectivity*)

> Topological index - The numerical basis for topological indices is provided (depending on how a molecular graph is converted into a numerical value) by either the adjacency matrix or the topological distance matrix. In the latter the topological distance between two vertices is the number of edges in the shortest path between these.

United Atom Approach - The united atom approach is a simplification used by molecular mechanics programs such as AMBER and CHARMM which approximates the influence of groups of atoms or molecular fragments by treating them as single atoms.

Verloop STERIMOL Parameters - The STERIMOL parameters defined by Verloop are a set of substituent length and width parameters (39).

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PCILO Calculations - PCILO (Perturbative Configuration Interaction using Localized Orbitals) calculations are semi-empirical molecular orbital calculations related to *CNDO/2* and *MNDO* calculations.

PDB - The Protein Data Bank (*PDB*) maintained at Brookhaven National Library, Upton, New York, which contains X-ray structures of several hundreds of proteins. *(see PDB file)*

PDB File - A *PDB* (Protein Data Bank) file is an *ASCII* (*American Symbolic Code for Information Interexchange* = text) file used to store the atomic coordinates of a molecule, usually a protein or nucleic acid. *(see PDB)*

Pharmacophore Generation - Pharmacophore generation is a procedure to extract the most important common structural features relevant for a given biological activity from a series of molecules with a similar mechanism of action.

PM3 - PM3 is a widely used semi-empirical molecular mechanics program. *(see Molecular mechanics)*

Principal Components Analysis (PCA) - Principal components analysis is a data reduction method using mathematical techniques to identify patterns in a data matrix. The main element of this approach consists of the construction of a small set of new orthogonal, i.e., non-correlated, variables derived from a linear combination of the original variables.

Principal Properties - Principal properties are scales of substituent or amino acid values derived by principal components analysis from a large matrix of structure descriptor variables, and useful in series design and data analysis.

Quantitative Structure-Activity Relationships (QSAR)* - Quantitative structure-activity relationships (QSAR) are mathematical relationships linking chemical structure and pharmacological activity in a quantitative manner for a series of compounds. Methods which can be used in QSAR include various regression and pattern recognition techniques.

QSAR is often taken to be equivalent to chemometrics or multivariate statistical data analysis. It is sometimes used in a more limited sense as equivalent to Hansch analysis. QSAR is a subset of the more general term SPC (12).

Quantum Chemical Calculations - Quantum chemical calculations are molecular property calculations based on the Schrödinger equation, which take into account the interactions between electrons in the molecule.

Receptor* - A receptor is a protein or a protein complex in or on a cell that specifically recognizes and binds to a compound acting as a molecular messenger (neurotransmitter, hormone, lymphokine, lectin, drug, etc). In a broader sense, the term receptor is often used as a synonym for any specific (as opposed to non-specific such as binding to plasma proteins) drug binding site, also including nucleic acids such as DNA.

Receptor Mapping* - Receptor mapping is the technique used to describe the geometric and/or electronic features of a binding site when insufficient structural data for this receptor or enzyme are available. Generally the active site cavity is defined by comparing the superposition of active to that of inactive molecules.

Regression Analysis - Regression analysis is the use of statistical methods for modeling a set of dependent variables, Y, in terms of combinations of predictors, X. It includes methods such as multiple linear regression (MLR) and partial least squares (PLS).

Semi-Empirical Methods - Semi-empirical methods are molecular orbital calculations using various degrees of approximation and using only valence electrons.

> Semi-empirical quantum mechanical methods - The methods which use parameters derived from experimental data to simplify computations. The simplification may occur at various levels: simplification of the Hamiltonian (e.g. as in the Extended Hückel method), approximate evaluation of certain molecular integrals (see, for example, zero differential overlap), simplification of the wave function (for example, use of p electron approximation as in Pariser-Parr-Pople).

Sequential Simplex Method - The sequential simplex method is an experimental design method used for the rapid optimization of properties.

SIMCA - The SIMCA (SIMple Classification Analysis or Soft Independent Modeling of Class Analogy) method is a pattern recognition and classification technique (36).

Simulated Annealing - Simulated annealing is a procedure used in molecular dynamics simulations, in which the system is allowed to equilibrate at high temperatures, and then cooled down slowly to remove kinetic energy and to permit trajectories to settle into local minimum energy conformations.

Slater-Type Orbitals (STO) - Slater-type orbitals are mathematical functions involving exponential functions, used in *ab initio* quantum chemical calculations. These functions mimic the electronic distribution in atoms and were used in *ab initio* calculations, but have now been superceded by Gaussian-type orbitals. *(see Gaussian-type orbitals)*

> Slater type atomic orbital (STO) - The exponential function on an atom; its radial dependence is given by $Nr^{n-1}\exp(-\zeta r)$, where n is the effective principal quantum number and ζ is the orbital exponent (screening constant) derived from empirical considerations. The angular dependence is usually introduced by multiplying the radial one by a spherical harmonic $Y_{lm}(\theta,\Phi)$.

SMILES - SMILES (Simplified Molecular Input Line Entry System) is a string notation used to describe the nature and topology of molecular structures.

Solvent-Accessible Surface - The solvent-accessible surface is described as the surface traced out by of a probe molecule, e.g., water, rolling over the Van der Waals surface of a molecule. There are two types: a) the surface formed by the locii of the centre of a spherical probe rolled around a molecule in the Van der Waals contact and b) the contact surface (or Connolly/Richards surface). *(see Connolly surface)*

STO-3G Basis Set - A STO-3G basis set is a set of Gaussian-type orbitals (*GTO*), each of which uses three Gaussian functions to approximate a Slater-type orbital (*STO*). More extended modern basis sets include STO-3-21G or STO-KG.

Structure-Based Design* - Structure-based design is a design strategy for new chemical entities based on the three-dimensional (*3D*) structure of the target

Molecular Shape - The molecular shape is an attribute of a molecule dealing with spatial extension, form, framework, or geometry. It is often described by, e.g., principal axes, ovality, or connectivity indices.

Molecular (Dis-)Similarity - Molecular (dis-)similarity is a number to express structural relatedness between pairs of molecules, e.g., the so-called Carbo, Hodgkin or Tanimoto coefficient (31,32).

Molecular Topology - Molecular topology is the description of the way in which the atoms in a molecule are bonded together. (*see Molecular connectivity, Topological index*)

Molfile - A molfile is a table containing atom type, connectivity and a more or less arbitrary *2D* or *3D* information about a molecule. Well-known file formats include the MOLfile used by MDL Information Systems Inc. (e.g., in the database MACCS), the MOL2 file used by Tripos Associates (e.g., in the modeling package SYBYL), or the CSSR format.

Monte Carlo Technique - The Monte Carlo technique is a simulation procedure consisting of randomly sampling the conformational space of a molecule.

Mulliken Population Analysis - Mulliken population analysis is a method for allocating electrons to atoms in order to generate partial atomic charges. The results are strongly dependent on the basis set used.

> Mulliken population analysis - A partitioning scheme based on the use of density and overlap matrices of allocating the electrons of a molecular entity in some fractional manner among its various parts (atoms, bonds, orbitals). As with other schemes of partitioning the electron density in molecules, Mulliken population analysis is arbitrary and strongly dependent on the particular basis set employed. However, comparison of population analyses for a series of molecules is useful for a quantitative description of intramolecular interactions, chemical reactivity and structural regularities.

Multivariate Statistics - Multivariate statistics is a set of statistical tools to analyze data (e.g., chemical and biological) matrices using regression and/or pattern recognition techniques.

Neural Networks - *(see Artificial neural networks)*

Non-Bonded Energy Terms - Non-bonded energy terms are potential energy functions describing Van der Waals, electrostatic and hydrogen bonding interactions in a force field.

Parameter Space - The parameter space is a multidimensional space spanned by the descriptors in a data set.

Partial Least Squares (PLS) - Partial least squares projection to latent structures (*PLS*) is a robust multivariate generalized regression method using projections to summarize multitudes of potentially collinear variables (33).

Pattern Recognition* - Pattern recognition is the identification of patterns in large data sets, using appropriate mathematical methodology. Examples are principal component analysis (*PCA*), *SIMCA*, partial least squares (*PLS*) and artificial neural networks (*ANN*) (3,34,35).

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GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
butyl flufenamate	topical antiinflammatory	1983	19, 316

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
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
cicletanine	antihypertensive	1988	24, 299
cidofovir	antiviral	1996	32, 306
cilazapril	antihypertensive	1990	26, 301

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
donepezil hydrochloride	anti-Alzheimer	1997	33, 332
dopexamine	cardiostimulant	1989	25, 312
dornase alfa	cystic fibrosis	1994	30, 298
dorzolamide HCL	antiglaucoma	1995	31, 341
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxifluridine	antineoplastic	1987	23, 332

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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
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
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
felbamate	antiepileptic	1993	29, 337
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenticonazole nitrate	antifungal	1987	23, 334
fexofenadine	antiallergic	1996	32, 307
filgrastim	immunostimulant	1991	27, 327

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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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
misoprostol	antiulcer	1985	21, 329
mivacurium chloride	muscle relaxant	1992	28, 334
mitoxantrone HCl	antineoplastic	1984	20, 321
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
modafinil	idiopathic hypersomnia	1994	30, 303
moexipril HCl	antihypertensive	1995	31, 346
mofezolac	analgesic	1994	30, 304
mometasone furoate	topical antiinflammatory	1987	23, 338
moricizine hydrochloride	antiarrhythmic	1990	26, 305
moxonidine	antihypertensive	1991	27, 330
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
mycophenolate mofetil	immunosuppressant	1995	31, 346
nabumetone	antiinflammatory	1985	21, 330
nadifloxacin	topical antibiotic	1993	29, 340
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306
naftifine HCl	antifungal	1984	20, 321
nalmefene HCl	dependence treatment	1995	31, 347
naltrexone HCl	narcotic antagonist	1984	20, 322
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
oxaliplatin	anticancer	1996	32, 313
oxaprozin	antiinflammatory	1983	19, 322
oxcarbazepine	anticonvulsant	1990	26, 307
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
paclitaxal	antineoplastic	1993	29, 342
parnaparin sodium	anticoagulant	1993	29, 342
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
pantoprazole sodium	antiulcer	1995	30, 306
paroxetine	antidepressant	1991	27, 331
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pegaspargase	antineoplastic	1994	30, 306
pemirolast potassium	antiasthmatic	1991	27, 331
penciclovir	antiviral	1996	32, 314
pentostatin	antineoplastic	1992	28, 334
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
pidotimod	immunostimulant	1993	29, 343
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide hydrochloride	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pimobendan	heart failure	1994	30, 307
pinacidil	antihypertensive	1987	23, 340
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
raltitrexed	anticancer	1996	32, 315
ramipril	antihypertensive	1989	25, 317
ramosetron	antiemetic	1996	32, 315
ranimustine	antineoplastic	1987	23, 341
ranitidine bismuth citrate	antiulcer	1995	31, 348
rebamipide	antiulcer	1990	26, 308
reboxetine	antidepressant	1997	33, 342
remifentanil HCl	analgesic	1996	32, 316
remoxipride hydrochloride	antipsychotic	1990	26, 308
repirinast	antiallergic	1987	23, 341
reteplase	fibrinolytic	1996	32, 316
reviparin sodium	anticoagulant	1993	29, 344
rifabutin	antibacterial	1992	28, 335
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
riluzole	neuroprotective	1996	32, 316
rimantadine HCl	antiviral	1987	23, 342
rimexolone	antiinflammatory	1995	31, 348
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rivastigmin	anti-Alzheimer	1997	33, 342
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin hydrochloride	hyperphenylalaninemia	1992	28, 336
saquinavir mesylate	antiviral	1995	31, 349
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
seratrodast	antiasthmatic	1995	31, 349
sertaconazole nitrate	topical antifungal	1992	28, 336
sertindole	neuroleptic	1996	32, 318
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline hydrochloride	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
simvastatin	hypocholesterolemic	1988	24, 311
sobuzoxane	antineoplastic	1994	30, 310
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatomedin-1	growth hormone insensitivity	1994	30, 310
somatotropin	growth hormone	1994	30, 310
somatropin	hormone	1987	23, 343
sorivudine	antiviral	1993	29, 345
sparfloxacin	antibiotic	1993	29, 345
spirapril HCl	antihypertensive	1995	31, 349
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
temocapril	antihypertensive	1994	30, 311
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terbinafine hydrochloride	antifungal	1991	27, 334
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333
tiagabine	antiepileptic	1996	32, 319
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
tilisolol hydrochloride	antihypertensive	1992	28, 337
tiludronate disodium	Paget's disease	1995	31, 350
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324
tiopronin	urolithiasis	1989	25, 318
tiquizium bromide	antispasmodic	1984	20, 324
tiracizine hydrochloride	antiarrhythmic	1990	26, 310
tirilazad mesylate	subarachnoid hemorrhage	1995	31, 351
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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

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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

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alclometasone dipropionate	ANTIINFLAMMATORY,	1985	21, 323
aminoprofen	TOPICAL	1990	26, 298
betamethasone butyrate propionate		1994	30, 297
butyl flufenamate		1983	19, 316
deprodone propionate		1992	28, 329
felbinac		1986	22, 320
halobetasol propionate		1991	27, 329
halometasone		1983	19, 320
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