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SECTION I. CNS AGENTS

Editor: John M. McCall, The Upjohn Company
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Chapter 1. Sigma Receptors and their Ligands: The Sigma Enigma

Magid Abou-Gharbia
Wyeth-Ayerst Research, CN 8000; Princeton, N.J. 08852

Seth Y. Ablordeppey and Richard A. Glennon
Department of Medicinal Chemistry, Medical College of Virginia/Virginia Commonwealth University; Richmond, Virginia 23298-0540

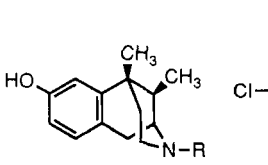
Introduction - The term *sigma (σ) receptors* was introduced in 1976 (1) to account for the actions of benzomorphan opioids such as (±)N-allylnormetazocine (NANM; SKF 10,047)(1). Initial interest in σ receptors was slow to develop and, as late as 1985, only 30 papers were published on this topic. However, there has since been steady growth in σ receptor research such that at the end of 1992 over 500 additional papers had appeared. And yet, after nearly two decades of research, little is known regarding the biochemical and molecular nature of these sites. The debate continues over whether σ sites are true receptors or a membrane bound enzyme. Although σ receptors have been implicated in the regulation of neurotransmitter release (2), known neurotransmitters do not bind at σ sites. However, evidence is now emerging that links σ receptor interaction with endogenous ligands (3,4). β-Endopsychosin, a low molecular weight polypeptide isolated from bovine brain extracts, and sigmaphens, possible endogenous ligands from guinea pig brain, inhibit σ binding (5). Neuropeptide Y (NPY) and peptide YY also bind at σ sites (3). An outstanding comprehensive review of σ receptors was published in 1990 (6). The present review will focus primarily on work published since that time.

PROPERTIES AND NATURE OF SIGMA BINDING SITES

The original classification of opiate receptors singled out NANM-related agents (e.g., 1-3) because they produced a psychotomimetic effect in humans and a spectrum of effects in the dog referred to as canine delerium. Although NANM binds at μ and κ opiate receptors, its effects could not be attributed to actions at these sites. Furthermore, whereas (-)NANM binds with higher affinity than its (+)enantiomer at other opiate receptors, its enantioselectivity is reversed at σ receptors. Over the years, the definition of σ receptors has undergone considerable change. Because the original σ ligands were benzomorphans, the binding sites were initially considered a type of opiate (i.e., σ-opiate) receptor. It was later thought, because NANM binds at PCP (phencyclidine) sites, that the two sites were identical (i.e., PCP/σ sites). Although this was somewhat gratifying because some NANM-related agents are psychotomimetic, it was quickly noted that the psychotomimetic effects of these benzomorphans are associated primarily with the levo and not the dextrorotatory isomers which display higher affinity for σ sites (7). However, due to differences in brain localization, and because certain non-benzomorphans (e.g., haloperidol; 4) displayed different affinities for these sites, new terms, such as haloperidol-sensitive σ sites, were adopted. More recently, subpopulations of σ sites have been proposed.

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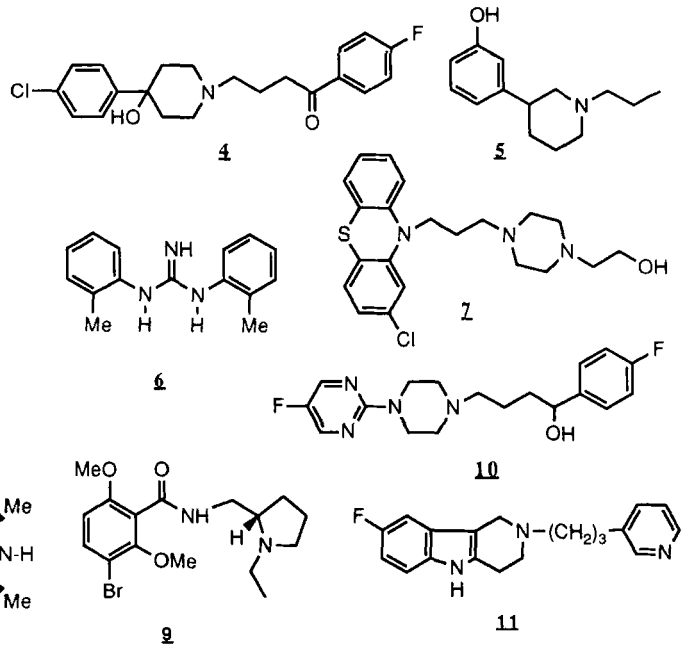
Perhaps the most significant reasons contributing to the slow development of this field is a lack of selective high-affinity ligands, and a lack of functional assays that can adequately characterize σ pharmacology. Long considered a prototypical σ ligand, NANM, for example, binds only with modest (Ki = 80 - 800 nM) affinity at σ sites and its pharmacological effects may be confounded by its actions at μ, κ, and PCP sites (6). Other ligands widely used to study σ receptors include haloperidol and N-*n*-propyl-3-(3-hydroxyphenylpiperidine (3-PPP; 5); however, these agents also lack selectivity. Haloperidol is a classical D2 antagonist. 3-PPP has been shown to behave as a dopamine receptor agonist, as an inhibitor of monoamine uptake, and possibly acts at adrenoceptors or some other non-σ receptor (8). Another widely used σ agent is N,N'-di-(*o*-tolyl)guanidine (DTG; 6). DTG, although lacking significant affinity for PCP sites, has been



1. R = $CH_2CH{=}CH_2$

2. R = $CH_2CH{=}C(CH_3)_2$

3. R = CH_2-cyclopropyl

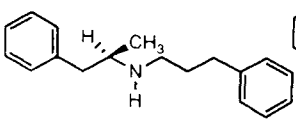


demonstrated to produce PCP-like effects in animals (9). Other problems contributing to the confusion surrounding σ research are the use of different radioligands to label σ sites (e.g., tritiated (+)NANM, (+)pentazocine (2), haloperidol, (+)3-PPP, DTG), and the use of different animal species for binding studies (e.g., rat, mouse, guinea pig, sheep, pigeon). Until recently, tools to properly investigate σ receptors simply have been unavailable. Indeed, many of the agents that helped generate interest in σ receptors bind only with micromolar affinities. Recently, agents with significantly higher affinities have been identified leading to the introduction of the term "superpotent σ ligands" – agents that bind at σ sites with subnanomolar affinities (10). One final problem, related to a lack of functional assays, is the inability to adequately categorize agents as being σ agonists or σ antagonists. Although NANM, 3-PPP, and DTG are considered σ agonists, and haloperidol an antagonist, these designations are not without controversy (6).

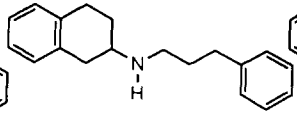
Sigma receptors appear to be concentrated in the brain stem, in certain limbic structures, in sensory areas, and in brain regions associated with endocrine function (6). They are also found in the periphery: for example, in rat liver (11,12) and heart (13), guinea pig ileum (14), blood vessels of several species of animals (8,15), on mouse lymphocytes and thymocytes (16), and in rat, mouse, and guinea pig vas deferens (but see 17).

SIGMA LIGANDS

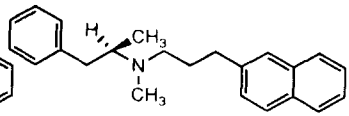
Neuroleptic agents - A large number of structurally diverse compounds have been examined as potential σ ligands. Like haloperidol, many potent neuroleptic agents such as perphenazine (7) bind at σ sites. Atypical antipsychotic agents such as rimcazole (8), remoxipride (9), BMY 14,802 (10) and gevotroline (11) also bind at σ sites (18-20). Many of the published studies represent isolated observations; few detailed structure-affinity studies have been reported.



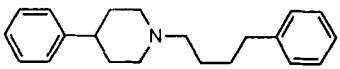
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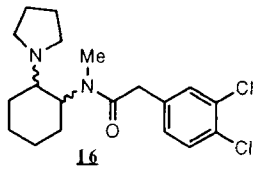
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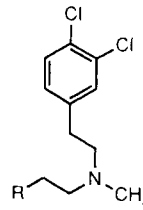
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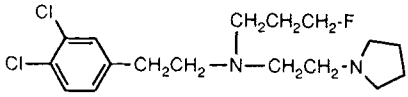


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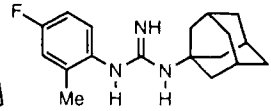


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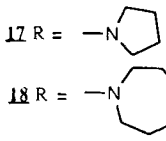




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17 R = —N

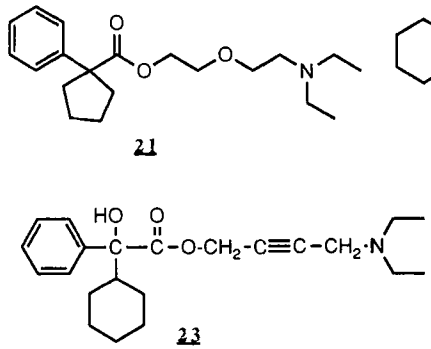
18 R = —N

Benzomorphans and Phenylalkylamines - Benzomorphans such as 1-3 were considered the first σ ligands. It has been demonstrated that N-substituted phenylethylamines may constitute the primary pharmacophore for NANM-related benzomorphans (21); this pharmacophore is also present in 3-PPP (22). R(-)PPAP (12), for example, binds with about 20 times the affinity of NANM (Ki = 429 nM) at [^{3}H]DTG-labeled σ sites (21,23). Its conformationally restricted analog 13 binds with identical affinity (22). Both 12 and 13 also bind with high affinity at 5-HT1A receptors suggesting there may be a certain amount of sequence homology between the two types of receptors that allows them to accommodate structurally related compounds (24). Several structural features were identified that significantly influence selectivity and 14, for example, binds with high affinity and > 400-fold selectivity over 5-HT1A receptors (24). Structure-affinity relationship (SAFIR) studies suggested that the phenolic hydroxyl group of the benzomorphans does not contribute to binding (21); this was supported by a recent investigation of several deshydroxy benzomorphan derivatives (25). Interestingly, whereas N-substituted phenylalkylamines typically display < 10-fold stereoselectivity at σ sites, the N-substituted benzomorphans bind with greater enantioselectivity suggesting that the rigid framework of the latter imparts directionality to the lone pair nitrogen electrons and/or that the steric bulk of the tricyclic system contributes to enantioselectivity (25). Extension of the 3-(phenyl)propyl group of S(+)PPAP to a 5-(phenyl)pentyl substituent resulted in a high-affinity sigma ligand. Its N-monomethyl derivative binds with yet higher affinity (21). Indeed, 5-substituted pentylamines reportedly represent a novel class of high-affinity (i.e., superpotent) σ ligands (26).

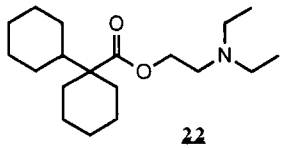
Haloperidol analogs - Haloperidol (4) binds at σ sites with high affinity (Ki ca 5-50 nM). To date, a detailed SAFIR study on haloperidol analogs has not been published. However, an investigation of 4-phenylpiperidines and 1-phenylpiperazines identified several novel haloperidol-related agents with subnanomolar affinities: for example, 15 (Ki = 0.8 nM; [^{3}H]DTG) (27). The 10-fold higher affinity of 15 over haloperidol suggests that certain of the electronegative substituents of haloperidol actually detract from σ binding (27).

Derivatives of U-50,488 - The κ-opiate agonist U-50,488 (16) (Ki = 81 nM; [^{3}H](+)3-PPP) represents another lead compound; structure-affinity studies resulted in superpotent compounds such as 17 and 18 (10,28,29). Compound 19 also exhibited high affinity for displacement of [^{3}H](+)3-PPP and [^{3}H](+)pentazocine (30).

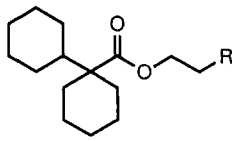
Guanidines - Guanidine derivatives represent yet another class of agents that has received considerable attention. The SAFIR of DTG-related compounds have been investigated in detail (31). The fluoro derivative 20 binds with 20 times the affinity of DTG, and the ^{18}F derivative was developed for positron emission tomographic studies (32).



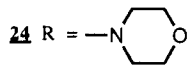
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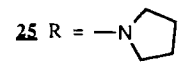
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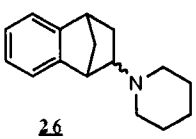


24 R = —N O

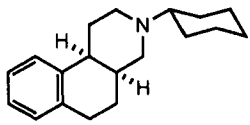


25 R = —N

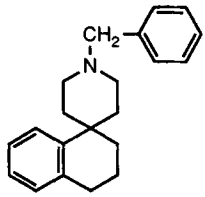
Newer compounds - Carbetapentane (21) binds with modest affinity at [^{3}H](+)pentazocine- and [^{3}H]DTG-labeled σ sites (33). Structurally related compounds also bind with Ki values ranging from about 5 to 50 nM: dicyclomine (22), oxybutynin (23) (33), PRE 084 (24), and PRE 079 (25) (34). A number of novel compounds, which may be alternately viewed as phenylalkylamines, 4-substituted piperidines, or conformationally restricted counterparts thereof, have also been reported. These include the superpotent 5,6-benzobicy-



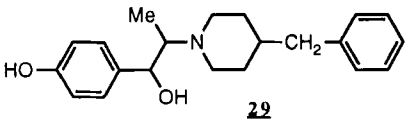
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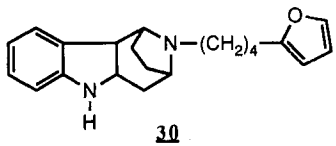
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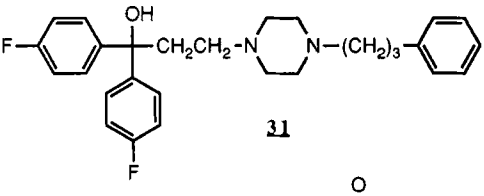
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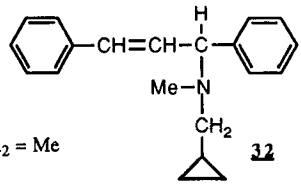
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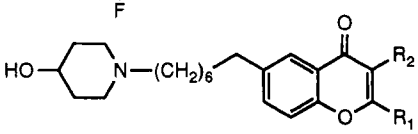
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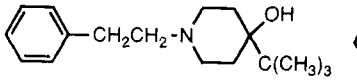


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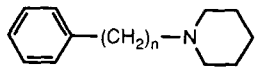


33 R_1 = Ph, R_2 = Me

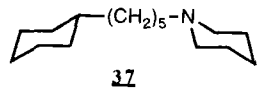
34 R_1 = Cyclohexyl, R_2 = H



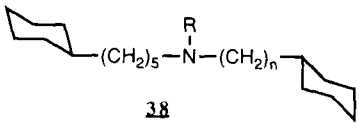
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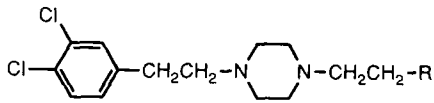
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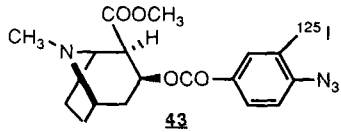


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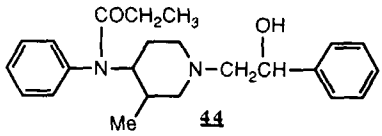




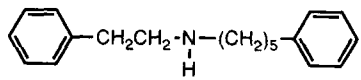
43

39 R = CH_3, 40 R = CH_2-F

41 R = $(CH_2)_3$-F, 42 R = CH_2-I



44



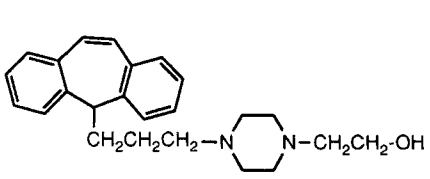
45

clo[2.2.1]heptane 26 (Ki = 0.075 nM; [^{3}H](+)3-PPP) (35), the benz[f]isoquinoline derivative 27, (36), the spiropiperidine 28 (37), and ifenprodil (29) (38). γ-Carboline derivatives 11 and 30 (39) may also be viewed as substituted piperidines. The potent dopamine uptake blocker GBR-12909 (31) binds with an affinity comparable to that of haloperidol (40). The cinnamyl derivative JO 1784 (32) also binds at [^{3}H](+)NANM-labeled σ sites (41).

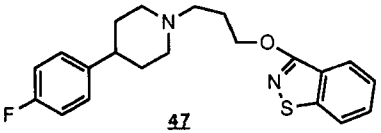
Aminoalkoxychromones were reported to be selective σ ligands. Compound 33 (IC50 = 51 and 55 nM at [^{3}H]DTG-and [^{3}H](+)3-PPP-labeled sites, respectively) binds at D2 dopamine sites with low affinity (Ki 21,000 nM). Compound 34 was systemically effective in behavioral models predictive of antipsychotic activity and in an animal model of ischemia (42).

Several modeling studies were conducted to determine the topography of σ receptors. Some of the original studies have been reviewed (6). More recently, the conformational and electrostatic properties of four σ ligands (haloperidol, (+)3-PPP, (+)pentazocine, and progesterone) were investigated (43). Molecular modeling studies were also conducted to develop a pharmacophoric model that accommodates benzomorphans (25). 3-D QSAR analysis of σ receptor ligands using comparative molecular field analysis (COMFA) led to a model that was applied to obtain quantitative estimates of σ binding affinities (44). The model was subsequently used to explain structural relationships between the binding of haloperidol and butaclamol derivatives, and to design several butaclamol-related analogs. Compound 35, for example, binds with high affinity and extension of the alkyl chain and removal of the hydroxyl and t-butyl groups further enhance affinity (i.e., 36 n = 5 and 8, Ki < 2 nM) (45). Reduction of the aromatic ring, as in the superpotent derivative 37 (45), confirms earlier observations with 38 (n = 1 - 4) (46) that an aromatic moiety is not required for the binding of these types of compounds at σ sites.

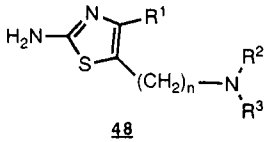
Compound 39 was synthesized as a template for the development of PET and SPECT compounds 40, 41 and 42 (30). Iodoazidococaine (43) was reported as a potent and highly specific photoaffinity label for



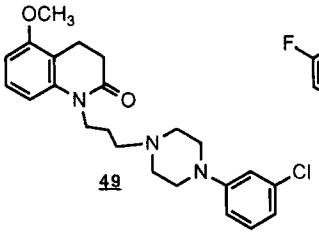
46



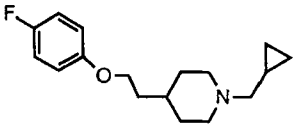
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σ sites (47). Tritiated ohmfentanyl (44) labeled both μ-opiate and σ binding sites (48), and the tritiated antidepressant opipramol (46) labeled two distinct sites (one of which appears to be a σ receptor) in rat brain membranes (49). Although ifenprodil (29) binds at σ sites (38), [^{3}H]ifenprodil seems to primarily label NMDA sites (50). New compounds disclosed in the patent literature included 47-50; analogs of 47 bind with Ki values of 0.02 - 7.3 nM (51), derivatives of 48 (n = 2 - 3) are disclosed as potential antipsychotic agents (52), compound 49 is claimed to be useful in disorders of consciousness (53), and 50 enhanced the antipsychotic effect of haloperidol in a conditioned avoidance paradigm (54).

SUBPOPULATIONS OF SIGMA RECEPTORS

Early pharmacologic studies suggested the possibility of multiple populations of σ binding sites (6). Evidence for heterogeneity continues to mount from functional (e.g., 14,55) and radioligand binding studies. [^{3}H](+)3-PPP binding in guinea pig brain fits a two-site model with Kd values for (+)3-PPP of 25 and 900 nM; [^{3}H]dextromethorphan (DM) labels at least two different sites (DM_1, DM_2) and the high-affinity DM_1 site seems to correspond to a σ site (7,56). With the availability of [^{3}H](+)pentazocine (57), it was shown that two σ sites can be differentiated: σ1 and σ2 (58). (+)Pentazocine binds with high affinity at σ1 sites and with much lower affinity at σ2 sites. DTG binds nearly equally well at both populations of sites. Furthermore, it has been suggested that σ1 sites may correspond to the above mentioned DM_1 sites (59). Rat heart σ sites may be of the σ2 type (13) whereas σ receptors in human cerebellum may be primarily of the σ1 type (60).

Table 1 provides some representative σ1 and σ2 binding data for various sigma ligands as reported by two groups of investigators. Both studies used [^{3}H]DTG in the presence of (+)pentazocine to label σ2 sites. In one study, σ1 data were obtained using [^{3}H]pentazocine (61), whereas the second study utilized [^{3}H]DTG as radioligand (59) and results were fit to a two-site model. Most σ ligands bind with higher affinity at σ1 sites than at σ2 sites; few agents bind with high affinity at σ2. Under ordinary assay conditions, the use of [^{3}H]DTG may not differentiate between σ1 and σ2 binding. The use of [^{3}H](+)3-PPP is more controversial. It has been suggested that this latter radioligand labels both sites (6) and yet some investigators contend that it primarily labels σ1 sites. Even the binding data in Table 1 are not in total agreement; for example, in one study (+)3-PPP displayed about 90-fold selectivity whereas in the other, it displayed only about 10-fold selectivity for σ1 sites. Using [^{3}H](+)pentazocine as radioligand (61), a series of phenylethylamines was found to bind with subnanomolar affinity; 45 (Ki = 0.17 nM), for example, binds with > 60 times the affinity of R(-)PPAP (12; Ki = 10.8 nM).

TABLE 1. Representative σ 1 and σ 2 binding data from two investigations.

Agent	σ1 Ki Value (nM)[a] (61)	(59)	σ2 Ki Value (nM)[a] (61)	(59)
Haloperidol	0.5	0.3	11	36
(+)Pentazocine	1.7	2.0	860	456
Carbetapentane	-	5.2	-	1523
Ifenprodil	5.8	-	2.3	-
Perphenazine	25	-	75	-
Fluphenazine	32	7.6	63	440
DTG	41	12	49	38
R(+)3-PPP	48	5	470	442
(-)Butaclamol	69	47	3200	3646
(+)Cyclazocine	-	78	-	1238
Buspirone	-	96	-	744
(-)Pentazocine	110	-	230	-
(+)NANM	150	45	15,000	4263
BMY 14,802	270	-	130	-
S(-)3-PPP	310	31	1200	1544
Remoxipride	310	-	1500	-
Chlopromazine	340	-	670	-
Dextromethorphan	520	121	14,000	53,503
(+)Butaclamol	580	-	1300	-
Rimcazole	1160	-	250	-
(-)NANM	3600	-	8600	-

[a] Binding data are from two different studies (59,61) that used different radioligands for labeling σ1 sites. The σ1 sites labeled in the second study (59) were referred to as DTG site 1. Ki values greater than 10 nM were rounded to the nearest whole number.

FUNCTIONAL AND CLINICAL RELEVANCE

The potential clinical utility of σ ligands has been reviewed (6,62-64). Early evidence suggested that σ ligands might constitute novel antipsychotic agents that lack the side effects associated with classical neuroleptic therapy (6,64,65). Autoradiographic and binding studies have shown the existence of σ binding sites in human brain (60,67), and post mortem examination revealed a reduction in the density of σ receptors in schizophrenia (68). A similar reduction was not observed in brain of subjects with chronic exposure to PCP (69). A review of σ antagonists as potential antipsychotic agents has been published (70). A relationship between the binding of cocaine analogs to σ receptors and their ability to induce psychosis has been observed (71) and a recent patent has appeared on the use of σ ligands for the treatment of cocaine abuse (54).

Sigma ligands may interfere with the uptake of norepinephrine, serotonin, and dopamine (8,15,17) and the release of acetylcholine (72). Although σ ligands may modulate NMDA receptors (55), NMDA receptor antagonists, but not σ ligands, disrupted learning in mice (73). Several σ ligands demonstrated a neuroprotective action in appropriate models (e.g., 74,75), and the neuroprotective effects of the antiischemic agent ifenprodil (29) have been suggested to involve σ receptors (38), as have those of opipramol (75). The potential role of σ ligands as neuroprotective agents has been reviewed (76).

The emetic response in pigeons may be σ-mediated (77). Sigma receptors may play a role in the actions of anticonvulsant agents (7,56) and in motor disorders (6). Various antidepressants, including tricyclic antidepressants and MAO inhibitors, bind at σ receptors (6). Although there has been some speculation that σ receptors may be involved in affective disorders (62), this has not been documented. The binding of certain antidepressants at σ sites may be related more to coincidental structural similarity between individual agents and σ ligands rather than to any mechanistic relevance. Due to the existence of σ binding sites on lymphocytes and thymocytes, it has been speculated that endogenous σ ligands may regulate immune homeostasis and/or immunocompetence (16).

In drug discrimination studies using rats trained to discriminate DTG from vehicle, stimulus generalization to several other σ-ligands (as well as to PCP and several other types of opioids) was observed; attempts to antagonize the stimulus with haloperidol and (-)butaclamol were unsuccessful (9). Although there is controversy as to whether the (+)NANM stimulus is σ mediated, a recent investigation provides support for at least some σ involvement (78). Other studies favored an NMDA-receptor mediated mechanism (79). Training dose could be a factor (78). In monkeys, the stimulus effects of (+)NANM may be mediated primarily by a PCP-based mechanism (80). However, in the rat, R(-)PPAP and several structurally-related agents lacking affinity for PCP receptors dose-dependently antagonized the actions of (+)NANM (81).

Conclusions - Sigma receptors may represent an entirely new mechanistic approach for the development of therapeutic agents useful in treating various mental, motor, and other disorders. The identification of novel, selective, and high-affinity ligands should aid in this process. Now that high-affinity (i.e., superpotent) σ ligands have been identified, the involvement of σ receptors in the actions of agents that bind only with micromolar affinities should be reexamined. But the problem of a lack of functional assays still exists. For example, a series of carbetapentane analogs with anticonvulsant activity has been recently identified; it is uncertain, however, if their mechanism of action involves σ receptors and if they are σ agonists or σ antagonists (82). It remains to be determined if all σ ligands bind at the same site. For example, an allosteric binding model has been proposed (6); this was subsequently modified to a "pseudoallosteric model" (59). It has been suggested that σ receptors exist in two discrete states having high and low affinity for σ ligands (83). Multiple populations of σ sites have also been described. Then there are the multiple DM binding sites, one (or more) of which may correspond to σ receptors. Does one type of σ (or DM) receptor correspond to a member of the cytochrome P-450 superfamily of enzymes (84)? Each of these areas needs to be addressed before further progress can be made in this field. It is perhaps for these reasons that some have referred to the situation as the sigma enigma (85).

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Chapter 2. Pharmacology of Neurotrophic Factors In Models of Neurodegenerative Disease

Peter S. DiStefano
Regeneron Pharmaceuticals, Inc.
Tarrytown, New York 10591

Introduction - The development of the nervous system depends in part on classes of molecules known as neurotrophic factors. The archetypal neurotrophic factor, nerve growth factor (NGF), was discovered some 45 years ago for its ability to support neurite outgrowth and survival of sympathetic and neural crest-derived sensory neurons. The recent cloning of additional NGF family members revealed the structures of brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), collectively known as the neurotrophins. Their structures and cognate high-affinity tyrosine kinase receptors, known as *trk*s, have recently been reviewed (1, 2). Another important neurotrophic molecule is ciliary neurotrophic factor (CNTF), which is related to the cytokine family of molecules. Although the mechanisms by which neurotrophic factors exert their actions are not completely understood, it has become increasingly evident that these factors have potential therapeutic benefit in the treatment of nerve trauma, toxic neuropathy and neurodegenerative disorders such as Alzheimer's disease, Parkinsonism, amyotrophic lateral sclerosis (ALS) and diabetes. Advances in biotechnology have allowed large-scale production of recombinant neurotrophic factors, permitting the study of these factors in various animal models of nervous system disorders. This review will summarize the in vitro actions of the neurotrophins and CNTF, as well as recent pharmacological studies of these factors in animal models of nerve trauma and neurodegeneration.

BIOLOGY OF NEUROTROPHINS

In vitro Actions - NGF has distinct survival and neurite outgrowth promoting activities on sympathetic and neural crest-derived sensory neurons of the dorsal root ganglion (DRG). However, sensory neurons derived from the neural placodes (as in the nodose ganglion) do not appear to require NGF for survival or process outgrowth (3). Ventral forebrain cholinergic neurons in the medial septum, diagonal band of Broca and nucleus basalis also respond to NGF by increasing choline acetyltransferase activity (4). The observation that NGF displays a limited spectrum of neurotrophic activities within the nervous system prompted the search for additional trophic substances, potentially related to NGF, that might subserve trophic functions on neuronal populations not responsive to NGF. Purification and cloning of the second neurotrophin, BDNF, revealed a molecule with trophic activities on neural crest-derived as well as neural placode-derived sensory neurons (5, 6). In addition, BDNF has trophic activity on motor, central forebrain cholinergic, nigral dopaminergic, hippocampal and striatal neurons, and on retinal ganglion cells (see rev. 7; Table 1). Homology cloning using conserved regions of NGF and BDNF as templates revealed yet a third neurotrophin family member, NT-3, that exhibits neuronal specificities overlapping with NGF and/or BDNF. However, its overall profile is distinct from the other neurotrophins (8-10; Table 1). A fourth member of this family was identified in *xenopus* and viper using PCR with degenerate oligonucleotide primers derived from sequences of the other neurotrophins (11). The human homologue of xenopus NT-4 was subsequently cloned and designated NT-4/5 (12, 13). NT-4/5 preferentially utilizes *trk*B as its cognate high affinity receptor yet its tissue distribution and biological spectrum is distinct from that of BDNF and other neurotrophins (13). The current status of the biological spectra of the four known neurotrophins on cultured embryonic neurons is outlined in Table 1 and emphasizes the point that these factors have overlapping as well as distinct patterns of activities, fulfilling the notion that multiple factors exists to subserve various trophic functions on the wide array of neurons within the peripheral and central nervous systems (PNS, CNS).

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Table 1. Neurotrophins and CNTF Affect Overlapping and Distinct Populations of Peripheral and Central Neurons

Neuronal Type	NGF	BDNF	NT-3	NT-4/5	CNTF
Peripheral					
Sensory (DRG)	+++	+++	+++	++	++
Sensory (Nodose)	-	++	+++	-	+++
Sympathetic	+++	-/+	-/+	N.D.	++
Parasympathetic	-	-	-	-	+++
Motor	-	+++	++	++	+++
Central					
Retinal Ganglion	-	++	+	N.D.	N.D.
Septal (cholinergic)	++	++	+	++	+
Hippocampal	-	++	++	++	++
Cerebellar	-	+	+++	+	++
Striatal (cholinergic/other)	+	++	+	++	-
Nigral (dopaminergic)	-	+++	+++	+++	-

+++, high activity; +, low activity; -, no activity; N.D., not determined. Arbitrary scores reflect survival-promoting or phenotype-altering actions of neurotrophins and CNTF.

Mechanism of Action - The mechanisms by which the neurotrophins exert their trophic actions are becoming increasingly clear, but gaps still remain. A scheme of neurotrophin receptor/signal transduction pathways involving ligand-stimulated autophosphorylation of *trk* receptors has recently been reviewed (14). In vivo, where neurons access neurotrophins at distal axons, far from the cell bodies, it is classically thought that neurotrophins are synthesized and constitutively released by neuronal targets, where they bind to high-affinity receptors, become internalized and are then retrogradely transported to the cell body (15-17). The recent observations that 1) BDNF and NT-3 mRNA (but not NGF mRNA) are localized to the cell bodies of adult DRG neurons and forebrain cholinergic neurons (18, 19), and 2) various neurotrophin receptors, including low-affinity NGF receptor (LNGFR) and *trk* A and B, are localized to the targets of sensory neurons, as well as the neurons themselves (20) suggest that, aside from their classical target-derived retrograde effects, neurotrophins may have paracrine or autocrine functions, affecting neuronal targets, as well as acting locally. Precisely what happens when neurotrophins arrive at the cell body is unclear, although NGF deprivation in sympathetic neurons results in metabolically-dependent or apoptotic cell death, a response that can be prevented by overexpression of the *bcl*-2 proto-oncogene (21, 22). Although retrograde transport is not proven as necessary for the actions of neurotrophic factors, it provides a means by which neurons can sample the events occurring at sites distal to the cell body, often several centimeters away. Retrograde transport of BDNF and NT-3 has recently been demonstrated in the PNS and CNS (23). Indeed, the patterns of retrograde transport correlate well with in vitro responses to the different factors, indicating that retrograde transport may predict neurotrophin responsiveness of adult neurons. This becomes important when determining which trophic factors have pharmacological potential towards a given compromised neuron.

NEUROPROTECTIVE EFFECTS OF NEUROTROPHINS

NGF - Administration of anti-NGF antibodies to neonatal rats results in immunosympathectomy (24), whereas administration of NGF itself causes hypertrophy and increased survival of developing sympathetic neurons that would have normally died as a result of competition for a limited supply of NGF (25, 26). These observations led to the hypothesis that NGF and other trophic substances "protect" neurons in the face of injury. Several studies that showed neuroprotective effects of trophic factors after toxic insult or neural trauma were initiated. For example, sympathectomy is readily induced in developing rats by administration of 6-hydroxydopamine. Co-administration of NGF prevents this sympathectomy (27). Similarly, NGF protects against the sympathectomy caused by colchicine and vinblastine, known inhibitors of microtubules that disrupt axoplasmic transport (28, 29). Additionally, the sympatholytic antihypertensive agent, guanethidine, causes sympathectomy when administrated to neonatal

rats and NGF protects against this effect by suppressing the aberrant expression of epitopes on sympathetic neurons caused by the guanethidine treatment (30, 31). These observations raise the possibility that trophic factors modulate immune interactions in the nervous system. In other models of toxic insult, NGF protects sensory neurons from capsaicin, the pungent moiety in hot peppers that causes permanent loss of small sensory fibers and functional sensory deficit (32). NGF also reportedly prevents the sensory neuropathies induced by the cancer chemotherapeutic agents taxol and cisplatin in adult rats (33, 34). Another intriguing observation is that NGF reduces the primary infection of sympathetic neurons by Herpes virus (35). In addition, NGF blocks the expression of latent Herpes virus in infected sympathetic neurons in response to stimuli that cause virus reactivation (36).

The observation that NGF (and more recently BDNF) is increased in the distal stump after peripheral nerve injury (37, 38), suggests that pharmacologic administration of NGF would effectively improve regenerative events. Indeed, regeneration of sensory axons through silicone chambers filled with NGF is accelerated compared to that in saline-filled chambers (39). The distal nerve Schwann cells appear to be active in regeneration processes by expression not only of trophic factors but also of enormously high levels of LNGFR (40). To this end, increasing the levels of distal nerve Schwann cell LNGFR enhances the rate of peripheral sensory nerve regeneration, possibly by binding and presenting increased levels of neurotrophin(s) (supplied in part by Schwann cells themselves) to regenerating axons (41). These studies indicate that neurotrophic factor receptor regulation also plays a critical role in repair of peripheral neurons. In the CNS, NGF can prevent the loss of cholinergic phenotype and cell number in medial septum/diagonal band neurons after a fimbria-fornix transection (42-44). NGF also appears to ameliorate cognitive deficits associated with this lesion (45). Based on these observations, NGF has been proposed as a potential therapeutic agent for the treatment of cognitive disorders such as Alzheimer's disease. However, this disease affects a plethora of monoaminergic and peptidergic neurons (eg., raphe, locus coeruleus) that do not appear to respond to NGF. In this light, other members of the neurotrophin family, with different or more widespread neurotrophic activities may be more favorable candidates to treat this disorder (see Table 1).

BDNF - *From Table 1 and earlier discussions,* BDNF clearly acts upon a greater variety of neurons than does NGF. Studies are now beginning to emerge on the pharmacological potential of BDNF (and to a lesser extent NT-3) in models of neuroprotection, nerve trauma and genetic disorders of the nervous system. BDNF has been shown to have trophic actions on dopaminergic neurons cultured from the developing ventral mesencephalon (46). In vivo, BDNF augments rotational behavior and nigro-striatal dopamine metabolism when administered *via* a cannula to the substantia nigra of normal adult rats (47), suggesting that BDNF is a trophic factor for central dopaminergic neurons in vivo. In vitro, BDNF protects dopaminergic neurons and related cell lines from both 6-hydroxydopamine- and N-methyl-4-phenylpyridinium ion (MPP^+)-induced cytotoxicity by increasing the activity of glutathione reductase and decreasing levels of oxidized glutathione (48). BDNF has also been tested for its ability to preserve nociceptive function in a mouse model of capsaicin-induced sensory neuropathy and was found to be as potent and as efficacious as NGF (49). Collectively, these results indicate that BDNF, like NGF, has cytoprotective effects on responsive neurons in the face of toxic insult.

From the standpoint of nerve trauma, investigators have recently shown that BDNF prevents spinal cord motor neuron cell death following sciatic nerve transection in neonatal rats (50). As well, cranial motor neurons of the facial nucleus are rescued by BDNF after common facial nerve transection (51). Although these studies were carried out in neonates, there is a strong rationale for BDNF and NT-3 effectiveness in lesioned adult motor neurons since these cells can retrogradely transport these two factors, but not NGF (23). Studies are currently in progress to determine the effect of BDNF on functional recovery of adult motor neurons after traumatic injury, as well as in mouse models of neuromuscular degeneration.

In the CNS, BDNF prevents the loss of cholinergic markers in the medial septum/diagonal band in response to fimbria-fornix transection (52, 53). BDNF was not as effective in this paradigm as NGF when both agents were administrated intracerobroventricularly; however, when both factors were administered intraparenchymally (at the site of trauma), BDNF was as effective as NGF, pointing to the importance of the sites of delivery of the different trophic factors within the CNS. As a behavioral correlate to these studies, intraseptal administration of BDNF was found to improve cognitive performance of rats after electrolytic lesion of the medial septal region (54). Studies on the effects of neurotrophins on CNS neuron sprouting and regeneration have been

initiated. Corticospinal tract neurons increase their sprouting response when administered NT-3 (but not BDNF or NGF) following a mid-thoracic spinal cord lesion. However, no effects of the neurotrophins are observed on regeneration of these neurons after axotomy (55). In an albino rat model of photoreceptor damage caused by exposure to constant fluorescent light, BDNF and NT-3, but not NGF, provide almost complete protection of the photoreceptor layer while minimizing the number of infiltrating macrophages in the retina (56). These studies implicate neurotrophins in the treatment of diseases of the eye including retinitis pigmentosa, macular degeneration and inherited retinal dystrophies.

CILIARY NEUROTROPHIC FACTOR

Biology - Ciliary neurotrophic factor was originally described for its trophic actions on parasympathetic neurons (57) but subsequent studies revealed trophic activities towards sensory, sympathetic, motor, cerebellar, hippocampal and septal neurons (58-62; see Table 1). CNTF is related to the cytokine class of molecules (leukemia inhibitory factor, LIF, and interleukin-6, IL-6) both in structure and receptor components (see below) and shows little identity to the neurotrophins (63). CNTF is highly localized to myelin-forming Schwann cells (64-66) as well as activated astrocytes, but levels in normal brain are moderate to low (67). The structure of CNTF further reveals the lack of a classical signal sequence, thus the mechanism of its cellular release remains obscure (68). An interesting feature of CNTF is its down-regulation in the distal sciatic nerve after injury (65, 66). High level CNTF expression in the periphery is not achieved until postnatal life, after the period of naturally occurring cell death (69), although exogenous application of CNTF to chicken embryos prevents naturally occurring cell death of motor neurons (70). CNTF is not localized to the targets of CNTF-responsive neurons in the way that NGF and the neurotrophins are (69). Based on these observations CNTF has been proposed as an "injury" factor, released by Schwann cells (by an unknown mechanism) only under pathological conditions (71). In support of this, retrograde axonal transport of CNTF has recently been characterized and shown to be dramatically increased in motor and sensory neurons after nerve injury (72). This observation is important as it provides a mechanism by which CNTF can access neurons within the CNS from the periphery.

CNTF Receptor - Recent advances have increased the understanding of the CNTF receptor structure and potential signal transduction cascades. The CNTF receptor complex is comprised of a CNTF binding protein, termed CNTFRα (73), and two other proteins, leukemia inhibitory factor receptor ß (LIFRß) and gp130 (74, 75). CNTFRα is not a transmembrane protein, but is anchored to the membrane through a glycosyl-phosphatidylinositol linkage (73). LIFRß and gp130 also serve as signal transducing receptor components for several other cytokines, including LIF, IL-6 and oncostatin M (76, 77). Whereas LIFRß and gp130 are expressed in a wide number of adult tissues, CNTFRα is expressed predominantly in neural tissues and skeletal muscle and identifies potential targets for CNTF action (78). Stimulation of cells expressing the tripartite CNTF receptor results in the rapid (minutes) phosphorylation of LIFRß and gp130 by a kinase(s) that is yet to be defined (75). CNTF also induces Tis-11 expression and other immediate-early genes in responsive cell lines and neurons with time courses characteristic for this class of genes (0.5-1 hr) (74). An intriguing observation is that cells containing LIFRß and gp130 can be rendered CNTF-responsive by the addition of soluble CNTFRα, suggesting that this normally GPI-anchored protein may be released and act on cells to promote responsiveness to CNTF (see rev. 63). Indeed, soluble forms of CNTFRα have been identified in cerebrospinal fluid, urine, and in culture supernatants of denervated muscle explants (79).

Pharmacology of CNTF - Given its potent effects on cultured motor neurons, several experimental paradigms have been established in vivo to determine CNTF efficacy on this class of neurons. CNTF prevents the axotomy-induced loss of facial motor neurons in neonates (80). As discussed previously, this is an effect shared by BDNF. However, CNTF treatment results in a more extensive preservation of neuronal number and phenotype. In the adult, CNTF has been tested in three genetic models of neuromuscular disorders, *wobbler, pmn* and *Mnd*. *wobbler* mice show a distinct and rapid deterioration of forelimb motor function and degeneration of corresponding motor neurons. This pathology is greatly obtunded by systemic treatment with CNTF (81). *pmn* (progressive motor neuronopathy) mice exhibit functional and morphological abnormalities similar to those observed in *wobbler*. CNTF treatment effectively ameliorates these deficits (82). In a third model, the *Mnd* (motor neuron disease) mouse, the histopathology is reminiscent of neuronal ceroid lipofuscinosis (83), but these animals experience motor impairment over a protracted time course (4-10 months). As in the *wobbler* and *pmn* models, CNTF administration

significantly improved motor performance in the *Mnd* mouse (84). These compelling in vivo observations serve as the rationale for currently ongoing clinical trials of CNTF in the treatment of ALS. In addition to its apparent effects at the motor neuron cell body, CNTF has also been shown to induce the sprouting of intact motor neurons upon direct application (85), further adding to its potential benefit to motor neurons during the course of disease or trauma.

Effects of CNTF on compromised CNS neurons have revealed that CNTF, like BDNF (see above), prevents photoreceptor degeneration in response to constant light exposure (56). As with BDNF, CNTF caused little if any macrophage infiltration into the retina. CNTF also prevents the axotomy-induced loss of central nervous system neurons in three separate models. First, intracerebroventricular infusion of CNTF prevents degeneration of medial septal neurons after fimbria-fornix transection without rescuing the cholinergic phenotype of these neurons (86). Interestingly, CNTF up-regulates LNGFR immunostaining in these neurons, suggesting that CNTF may regulate neurotrophin responsivity by altering the expression of neurotrophin receptor molecules. Second, CNTF prevents degeneration of nigro-striatal dopaminergic neurons after mechanical transection, again without rescuing the dopaminergic phenotype (tyrosine hydroxylase immunostaining) of these neurons (87). Third, CNTF prevents the axotomy-induced loss of neurons in anteroventral and anterodorsal thalamic nuclei (88). Taken together, these observations suggest that CNTF has neuroprotective effects on a broad range of neurons in both the PNS and CNS.

The cloning of CNTFRα and subsequent localization of its expression revealed a high level of CNTFRα in adult skeletal muscle (73), levels that increase dramatically after denervation of muscle (89). This raises the question whether muscle is a target for CNTF action. Recent studies have shown that CNTF partially prevents the denervation-induced atrophy of skeletal muscle, as assessed by wet weight, total protein and fiber type cross-sectional area (89). Interestingly, CNTF had no effect on any of these parameters in innervated muscle, supporting the above-mentioned hypothesis that CNTF acts primarily (though not exclusively) during the pathological state (71). These studies are of importance in that CNTF represents a trophic factor affecting not only neurons (eg., motor) but also the targets (muscle) of responsive neurons. This becomes important in diseases with both motor neuron and muscle pathology, such as nerve trauma and motor neuron disease (ALS).

Summary & Perspective - Rapid advances in the field of neurotrophic factors, and emerging animal studies, have brought hopes for novel therapies for a multitude of neurodegenerative disorders for which there is currently no treatment. Owing in great part to advances in biotechnology that allow the gram-kilogram scale production of these proteins, the pharmacology of neurotrophic factors can be elucidated. It is now important to establish "proof of principle" with these various neurotrophic factors in the clinical setting, showing that systemic administration of these polypeptide factors offers relief from various neurological disorders. Once this is established, it will be necessary to take advantage of second generation approaches such as chimeric trophic factors (90, 91) with "pan" neurotrophic activities, small molecule mimetics and effectors that strategically regulate neurotrophic factor receptors. With regard to CNS applications, there is the ever-present problem of delivering large polypeptides across the blood-brain barrier, thus it will be important to exploit blood-brain barrier-specific carrier systems (as recently described for NGF (92)), potentially novel signal transduction mechanisms, cellular transplant strategies and gene therapy approaches.

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Chapter 3. Neuronal BZD Receptors: New ligands, Clones and Pharmacology

Ronald B. Gammill and Donald B. Carter
Medicinal Chemistry Research and CNS Research
Upjohn Laboratories
Kalamazoo, Michigan 49001

Introduction - Drugs which interact at $GABA_A$ receptors do so at several distinct binding sites and allosterically modulate GABA-induced Cl^- flux. The affinity of these drugs, as well as their modulatory efficacy, changes with receptor composition (1). A report describing the identity of low affinity agonist binding sites for the $GABA_A$ receptor-chloride channel under conditions normally used in $^{36}Cl^-$ uptake assays has appeared (2). A review of heterocyclic ligands for the benzodiazepine receptor (BZR) describes the discovery of benzodiazepines in food products (3). The rational design and synthesis of haptens having specific activity as full agonists or full antagonists at the BZR has been reported (4). Molecular models for recognition and activation at the benzodiazepine receptor have been reviewed (5), along with additional reviews on the interpretation of the behavioral effects of BZR ligands (6), novel anxiolytics that act as partial agonists at BZRs (7), and the recently developed ligands for neuronal BZRs and their pharmacological activities (8) have appeared since the last report in this series.

Pharmacology of $GABA_A$ Receptor Subtypes - Over the past few years, a large body of knowledge regarding the molecular composition of $GABA_A$ receptors has been published. Consistent with other members of the ligand-gated ion channel family (9,10), the $GABA_A$ receptor is probably assembled as a pentameric structure from four possible subunit families. The subunit stoichiometry for any $GABA_A$ receptor complex is unknown. In the rodent there are currently six α-subunits (α1-α6), three β-subunits (β1-β3), three γ subunits (γ1-γ3) and a δ-subunit (11,12). Different subunit combinations confer distinct pharmacologies when those $GABA_A$ subunits are coexpressed in either frog eggs or animal cells. The γ-subunit class appears to confer a robust benzodiazepine (BZD) responsiveness to any α/β subunit combination (13,14). However, studies on $\alpha\beta\gamma$ combinations reveal that members of the α class have great influence on the BZD pharmacology associated with those trisubunit $GABA_A$ receptor subtypes (13,15). The α1-containing subtypes have relatively high affinity for CL 218-872 and β-carbolines whereas complexes containing α2, α3, and α5 show approximately 10 fold lower affinity for CL 218-872 and β-carbolines (15,16). However, α1, α2, α3, and α5 containing subtypes have a high affinity for the BZD antagonist Ro 15-1788 and the partial inverse agonist Ro 15-4513. The α4 and α6 subunits in combination with β2γ2 subunits bind Ro 15-4513 at a site insensitive to diazepam (17,18) which can be mutated to a diazepam sensitive site by substituting the amino acid histidine for arginine in α6(arg101), further demonstrating the crucial role the α subunits play in determining differential pharmacology (19). The γ subunits also contribute to the differential pharmacology of the $GABA_A$-BZD receptor complex since receptors composed of α1β1γ1 subunits have very low affinity for antagonists or inverse agonists (20). The γ3 subunit also imparts a unique BZD pharmacology to α1$\beta\gamma$ combinations. The ratios of affinities of γ3 to γ2 containing receptors is close to unity for inverse agonists and antagonists of the BZD modulatory binding site on $GABA_A$ receptors, but this ratio increases considerably for BZD site agonists (12). The effects of mixing various βs with α1γ2 appears not to greatly influence the modulation of diazepam or bretazenil on GABA mediated Cl^- currents in reconstituted $GABA_A$ receptors (21). Although the δ subunit is closely related by sequence to the $GABA_A$ receptor subunits, when it is substituted for γ2 in $\alpha\beta\gamma$ receptors, the ability of those receptors to bind BZDs is severely diminished. The δ subunit mRNA is found in areas of the brain which suggest that they function in GABA mediated neurotransmission but not in interaction with BZDs (22).

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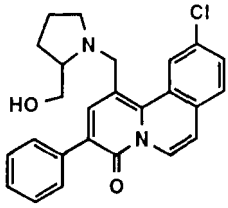
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Localization of Possible $GABA_A$ Subtypes in Rodent Brains - During the past several years, the sites of gene expression of most of the subunits have been partially identified by in situ hybridization. Recently, a systematic comparison of the 13 currently known rat $GABA_A$ receptor subunit transcripts using unique oligonucleotide probes specific for each subunit has been accomplished (23-25). The results of these studies are consistent with and extend a number of prior studies which were focused on several subtypes or a particular brain region (26-29). Colocalization of subunit mRNA suggests the possible formation of $GABA_A$ receptor subtypes, although these inferences are based on the assumption that mRNA levels reflect protein levels. However, this assumption has only tenuous support from immunocytochemical experiments with a small number of specific antibodies (29,30). The protein levels of the δ subunit codistribute with the mRNA levels (22) but γ2 mRNA levels show some discordance with γ2 immunoreactivity (30). A possible explanation for discordance between mRNA and protein would be that mRNA resides primarily in soma distal to dendrites or axon terminals where protein likely resides. The α1β2γ2 Type I complex appears to be the most predominately expressed receptor in rodent brain and is found in the cortex layers II/III and V/VI and cerebellum and at lower levels in some basal nuclei, midbrain septal nuclei and colliculi. Subtypes comprised of α2β2γ2 or α2β3γ2 are also widely distributed but highly expressed in layer II/III cortex, pyriform cortex, hippocampus, ventromedial hypothalamic nuclei and spinal cord where α3β2/3γ2 subtypes are primarily found. The possible subtypes containing the α4 subunit are suggested to be α4β2δ, α4β2, or α1α4β2δ combinations which are located in thalamic and hippocampal nuclei (23). However recent data suggests that the combination α4β2γ2 does not exist in vivo because Ro15-4513 binding could not be detected in thalamic areas where the α4 subunit is highly expressed (31). The α5β2γ2 subtype appears to be primarily a hippocampal subtype which shares much common BZD pharmacology with the α2 and α3 βγ combinations except for its very low affinity for the imidazopyridine zolpidem (16,23). The α6 subunit is found only in the cerebellar granule cells in combination with a number of other subunits; α1, β2, β3, γ2, and δ subunits. Thus the combinations that are possible include α6β2γ2, α6β2δ, α6δ etc. The exact combinations are unknown, but recent data suggest that a mutation in α6 at position ARG(100) to a GLN will confer diazepam binding on the α6β2γ2 subtype which may explain the changes in the susceptibility of the strain of rat carrying the mutation to sedation by exposure to ethanol (32). The possibility also arises that dual subunit combinations (such as α1γ2) can also give rise to BZD binding sites in vitro (33). This observation, of course, complicates the interpretation of in vivo drug action since there is also evidence that such dual subunit combinations may exist in vivo (34).

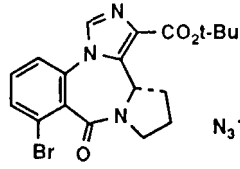
BZD Ligands - Four BZR ligands (triazolam, diazepam, Ro 19-8022 (**1**) and bretazenil (Ro 16-6028, (**2**)) were pharmacologically characterized in various neurological and behavioral paradigms in mice. Fractional BZR occupancy in vivo was assessed and correlated with the pharmacological effects. Diazepam and triazolam produced effects beginning at low to intermediate fractional BZR occupancy whereas **1** and bretazenil required a higher BZR occupancy to do so (35). A number of benzodiazepine receptor ligands with different chemical structures and different intrinsic pharmacological profiles were surveyed for their ability to displace [^{3}H]Ro 15-4513 (**3**) binding in rat cerebellar membranes. **3** is a negative modulator at the BZD receptor and binds with high affinity to all known BZD binding sites associated with the $GABA_A$ receptors. The results suggest that the cerebellar diazepam-insensitive **3** binding sites have unique substrate specificity (36). Compound **3** can be classified as a "GABA-neutral" (antagonist) ligand at DI and "GABA negative" (inverse agonist) at other BZRs. These findings suggest that $GABA_A$ receptor subunit composition determines not only ligand affinity but also ligand efficacy (37). Chronic administration of lorazepam reduces the coupling between the benzodiazepine agonist site and the chloride channel and concomitantly increases coupling between the channel and the inverse agonist site. These findings offer neurochemical evidence for cross-tolerance to ethanol and phenobarbital after induction of lorazepam tolerance (38). Alfaxalone, pentobarbital and diazepam potentiate γ-aminobutyric acid-induced depolarizations in single myenteric neurons. The data indicate that $GABA_A$ receptors on myenteric neurons contain binding sites for some steroids, barbiturates and benzodiazepines and that responses mediated at enteric $GABA_A$ receptors can be modified by drugs acting at these allosteric sites (39).

New Ligands for $GABA_A$ Subtypes - Changes in the substituents on N-5 and C-6 of the imidazoquinoxaline panadiplon (U-78875, 4) ring system produced no major effects on binding characteristics but yielded analogues of widely varying efficacy. The relative binding affinity and efficacy of the analogues measured in brain membranes were similar to those in cloned $GABA_A$ receptors of the $\alpha_1\beta_2\gamma_2$ (Type I) and $\alpha_3\beta_2\gamma_2$ (Type II) subtypes. The imidazoquinoxalines showed no marked subtype selectivity (40). The diimidazoquinazolines U-84935 (5), U-90167 (6), U-90168 (7) displayed various levels of efficacy as measured by their ability to potentiate GABA Cl^- currents in the $\alpha_1\beta_2\gamma_2$ subtype. The variations of the drug efficacy could be related to changes at the R_1, R_2 and R_3 substituents (41).

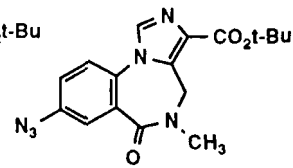
Compound 1 presents the neurological and behavioral profile of a typical partial agonist at the BZR exhibiting anticonflict effects in mice and rats with higher potency and efficacy than alprazolam. Its anticonvulsant potency and efficacy were comparable to alprazolam against either pentylenetetrazol, N-methyl-D-aspartic acid or maximal electroshock tests and it antagonized the motor impairment induced by diazepam or meclonazepam. This compound demonstrates both agonistic and antagonistic effects which is characteristic of compounds having intrinsic efficacies lying in between those of full agonists and pure antagonists (42,43).



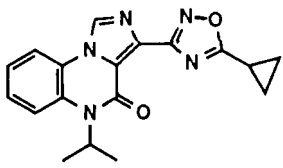
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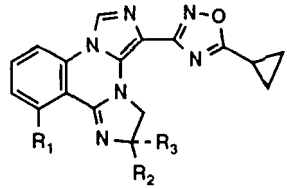
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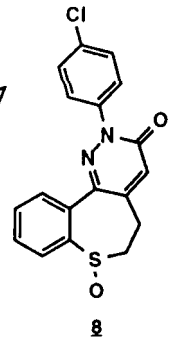
3



4



5 R_1=R_2=R_3=H
6 R_1=Cl; R_2=CH_3;R_3=H
7 R_1=Cl; R_2=H; R_3=CH_3



8

5,6-Dihydro-(1)-benzothiepino[5,4-c]pyridazin-3(2H)-one 7-oxide (Y 23684, 8) is a high affinity ligand (IC_{50} = 30 nM) that enhances GABA-induced chloride current in the isolated neuron with a low maximal effect. This latter effect was antagonized by flumazenil. The full effect of diazepam was antagonized by Y 23684 (44). This compound was anticonvulsant against seizures induced by bicuculline, leptazol or electroshock, but was clearly less active than diazepam in the latter two cases. The drug showed little or no sedative or muscle relaxant effects in a range of tests (45). Both enantiomers and racemic material had approximately equivalent in vivo activities to the bicuculline test in mice and conflict test in rats; however, the S-(+) enantiomer showed about three times higher affinity to the benzodiazepine receptor than the R-(-) enantiomer (46).

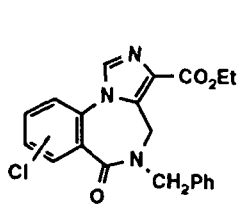
A series of imidazobenzodiazepin-6-ones (9) were synthesized and evaluated for their affinities at diazepam-sensitive (DS) and diazepam-insensitive (DI) benzodiazepine receptors in rat cortical and cerebellar membranes. Analogues with a N-5 benzyl group and C-7 or C-8 chlorine substituent demonstrated enhanced ligand affinity at the DS BZR (47).

RU 32698 (10) displaced ^{3}H-flunitrazepam binding (IC_{50} = 56 nM) and produced potential anxiolytic effects. It was less potent (compared to chlordiazepoxide) as an anticonvulsant and induced little or no sedation or muscle relaxation despite occupying virtually all in vivo BZD binding sites (48-50). Like several other partial agonists, it did not show tolerance to its anti-leptazol effect; possibly indicating less potential to induce dependence (51). It did not show the full sedative/muscle relaxant effects associated with full agonist BDZs in human volunteers, even at very high doses (500 mg) (52). Several members of this series tested in volunteers showed approximately the same ranking of sedative/muscle relaxant effects as observed in animal models (53,54). For example, RU 34000 (11) induced strong inverse agonist effects in vivo despite having low affinity for BZD receptors in vitro (IC_{50} = 980 nM). Kinetic studies on several members of this series suggest that the low binding affinity is compensated for by high drug levels in plasma and brain (55).

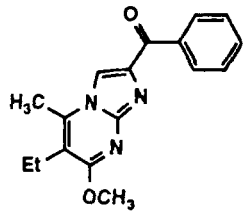
A report of the synthesis of imidazo[1,2-b]pyridazines, imidazo[1,2-a]pyridines, imidazo[1,2-a]pyrimidines and imidazo[1,2-a]pyrazines and their ability to displace [3H]diazepam from rat brain membrane has also appeared (56,57), along with a brief summary of the activity with imidazobenzthiazoles, including Ru 33872 (12) (6).

FG 8205 (13) is a partial agonist which did not protect against seizures induced by electroshock. It significantly antagonized the rotarod performance deficit induced by diazepam in the mouse and did not show marked motor impairment as seen with full agonist. High efficacy was observed in anticonvulsant and anxiolytic tests (58).

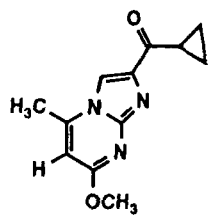
The contributions of BZR subtypes to the generation of the CL 218872 (14) cue has been reported (59). Ru 32698 and Ru 43028 (15) were less potent in substituting for the CI 218872 cue than was chloridiazepoxide.



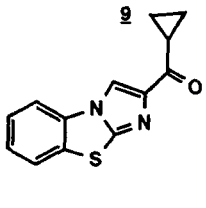
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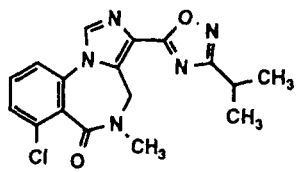
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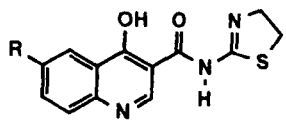
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13

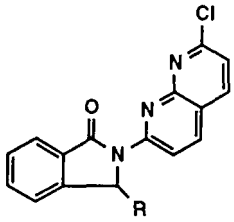


14 R=H
15 R=OCH3

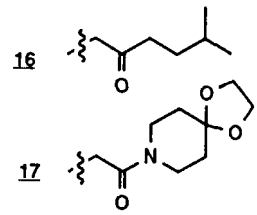
The cyclopyrrolones do not induce withdrawal signs after repeated administration and therefore may have less dependence liability (60). RP 59037 (16) induced a relatively small increase in TBPS binding in vitro and showed a non-sedative anxiolytic profile in vivo. It was effective in conflict and plus maze models of anxiety and antagonized seizures induced by leptazol and isoniazid but not those induced by strychnine. Little or no effects were observed in models detecting sedation or muscle relaxation. The partial agonist efficacy could also be further characterized by demonstrating antagonism of full agonists in these tests in which it is ineffective (61,62).

DN 2327 (17) demonstrated very high affinity for the BZRs (IC_{50} = 0.4 nM for displacement of 3H-diazepam). A low agonist-like shift of the BZR binding by GABA suggests a partial agonist. DN 2327 was effective in models of anxiety and was antagonized by flumazenil. The compound was anticonvulsant against seizures induced by leptazol or bicuculline but not those induced by electroshock. It was inactive in a range of tests detecting sedation or muscle relaxation (spontaneous locomotion, potentiation of pentobarbital or ethanol and rotarod and inclined screen tests). In general these actions are consistent with partial agonism for DN 2327. However, the same dose of DN 2327 potentiated the antileptazol effect of diazepam but attenuated the potentiation of pentobarbital induced by diazepam. As the dose of diazepam used in the two tests was also the same, these data are apparently inconsistent with the theory of partial agonism at one functional receptor type (62).

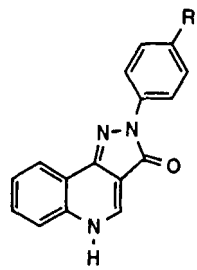
Thienylpyrazoloquinolines originated from a series of pyrazoloquinolines which includes the weak partial agonist CGS 9896 (19) and the weak partial inverse agonist CGS 8216 (18). The efficacies of these compounds have been closely defined and all the criteria for partial agonism have been satisfied. The thienylpyrazoloquinolines exhibited high affinity for the BZR. Different positional isomers of the thienyl ring showed different efficacies and, in all cases, increasing the size of the alkyl substituent on the thienyl ring shifted efficacy towards agonism. S-135 (20) is a strong parial inverse agonist which did not induce seizures alone but potentiated leptazol seizures. At the ED_{50} dose for potentiation of leptazol S-135 occupied a proportion of BZD receptors in vivo slightly more than DMCM (21) but considerably less than CGS 8216, suggesting strong partial inverse agonist efficacy. It antagonized pentobarbital induced anaesthesia in mice and monkeys and increased EEG arousal and spontaneous locomotion. It enhanced the crossed extensor reflex with a maximal effect similar to that induced by DMCM and βCCM (22). This latter effect was blocked by flumazenil. As with other inverse agonist, S-135 has potential memory enhancing properties (63).



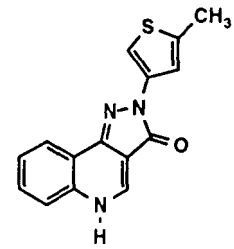
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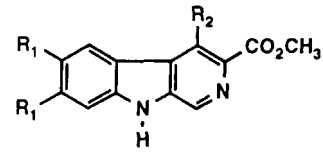
17



18 R=H
19 R=Cl



20

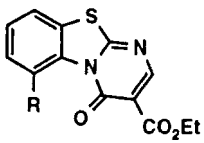


21 R_1=R_2=H
22 R_1=OCH_3; R_2=Et

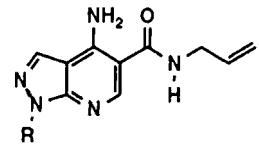
4H-Pyrimido[2,1-b]benzothiazol-4-ones bind to the benzodiazepine receptor with potency greater than chlordiazepoxide. Substitution at the 6-position resulted in compounds with highest affinity. The most active compounds were **23** and **24** (64).

The pyrazolopyridine ICI 190622 (**25**) showed affinity for the BZR (IC_{50} = 81 nM) in the cerebral cortex but higher affinity in the cerebellum (IC_{50} = 19 nM). In vitro measures and efficacy (GABA shift and photoaffinity labelling shift) suggest partial agonist efficacy. Its behavioral profile is also consistent with this hypothesis. It is effective in conflict models of anxiety in several species and is a potent anticonvulsant (leptazol and bicuculline) (65,66).

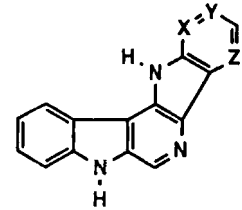
Azadiindoles, benzannelated pyridodiindoles and indolopyridoimidazoles (**26**, **27**, **28**) have been synthesized to define the spatial dimensions of the lipophilic regions of the benzodiazepine receptor binding cleft. Contributions to agonist, antagonist and inverse agonist activity as a function of structure were established (67). The synthesis of the novel 8H-diimidazo[1,5-a-2',1'-c][1,4] benzodiazepine, structurally related to bretazenil, has been reported (68).



23 R = CH_3
24 R = Cl



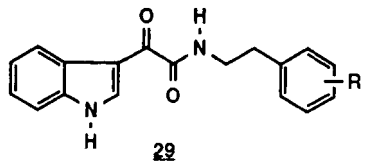
25
R = CH_2CH_2—≡—CH_3



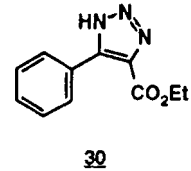
26 X=N; Y=CH; Z=CH
27 X=CH; Y=N; Z=CH
28 X=Y=CH; Z=N

Although the complete biosynthetic pathway of β-carbolines in animals and man is not known, it seems evident that these compounds are derivatives of tryptophan, tryptamine or more generally of a (2-aminoethyl)indole structure. In this report, tryptamine, tyramine and dopamine moieties are linked to the indole nucleus by an oxalyl bridge. Based on GABA ratios and in vivo tests, the most potent of these compounds (**29**) behave as inverse agonists at the BZR (69).

A series of substituted (phenyl, benzyl and pyridyl) 1,2,3-triazoles were prepared and tested for their ability to displace [^{3}H]diazepam from bovine brain membrane protein. Only one compound, 4-carboethoxy-5-(4-pyridylamino)-1,2,3-triazole (**30**), was found active (70).



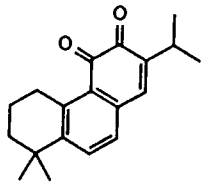
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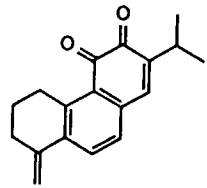
30

A series of analogues related to the natural product miltirone (**31**) have appeared. It is apparent from SAR studies that the A ring of **31** is essential for activity. However, the A-ring need not be a six membered ring since seven and eight membered rings also are well tolerated. MMLT (**32**) was found to be an antagonist while APMLT (**33**) appears to be a partial agonist (71,72). 5-O-Methyllicoricidin (**34**, from *Glycyrrhiza uralensis*) is a new potent benzodiazepine stimulator for the binding of BZD to rat synaptosomes (73). A report on the modulation of $GABA_A$-benzodiazepine receptor ligands in membranes from the rat forebrain by polyamines (spermine, spermidine and putrescine) has appeared (74). A series of nipecotic and guvacine analogues were prepared in which lipophilic groups were introduced onto the ring nitrogen. These modifications afforded

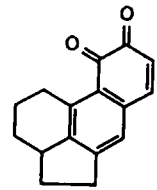
potent, orally-active anticonvulsants. Compound **35** was selected for extensive evaluation but produced severe adverse effects in Phase I clinical trials after single dose administration to humans (75).



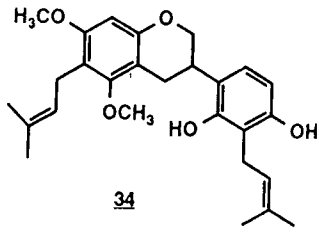
31



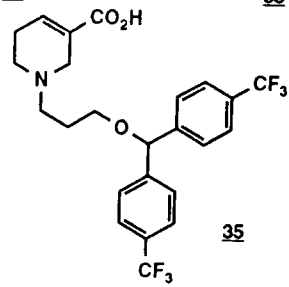
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34



35

A report has appeared where analogues of ZK 93426 were prepared to determine which functional groups are required for agonist activity. Several compounds exhibited high affinity for the BZR but in vivo studies failed to show anticonvulsant activity (76). A paper addressing the effects of the inverse agonist FG 7142 (N-methyl-β-carboline-3-carboxamide) and of the agonist diazepam of $GABA_A$ receptor subunit mRNA expression has appeared. The data show that chronic exposure to these compounds has opposing actions on levels of $GABA_A$ receptor subunit mRNA (77).

Given the rather specific pharmacological profile of the 3α-hydroxysteroids that are active at the $GABA_A$ receptor, it is quite plausible that safe and effective steroid anxiolytics, anticonvulsants and hypnotics can be developed (78). A report describing interactions between RU 5135 and the $GABA_A$-receptor complex has appeared. RU 5135 is a competitive $GABA_A$ receptor antagonist as measured by antagonism of GABA modulation of [^{35}S] TBPS at the $GABA_A$ receptor (79,80).

A QSAR study was carried out for the binding of a series of "classical" benzodiazepines and some β-carbolines with BZRs to investigate the active sites in the latter and the nature of the binding of compounds with them. The data supports the notion that BZR have many additional hydrophobic, hydrogen bonding and polar sites other than those previously suggested (81).

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Chapter 4. Molecular/Cell Biology of G-Protein Coupled CNS Receptors

Charles Gluchowski, Theresa A. Branchek, Richard L. Weinshank, Paul R. Hartig
Synaptic Pharmaceutical Corporation, Paramus, NJ

Introduction - The G-Protein Coupled Receptor (GPCR) superfamily contains well over 100 receptor proteins which play crucial roles in the regulation of numerous physiological processes in the central and peripheral nervous systems (1). Historically, a number of these receptors have been exploited as important targets for the design and development of significant therapies for CNS disorders such as psychosis (D_2 dopamine receptor antagonists such as haloperidol) and migraine ($5\text{-}HT_{1D}$ serotonin receptor agonists such as sumatriptan) (2,3). Recent advances in molecular and cell biology have supplemented classical pharmacological studies to provide a more precise understanding of the diversity, localization, nature and function of GPCRs (4). In support of drug design efforts, the preparation of heterologous expression systems containing individual cloned GPCRs in mammalian cell lines can yield very precise biological assays for structure activity relationship (SAR) studies (5,6).

Although the precise molecular structures of GPCRs are not known because these membrane bound proteins have not yielded to crystallization and x-ray diffraction studies, they appear to be similar in structure to the membrane protein bacteriorhodopsin which has been studied by electron diffraction: seven helical transmembrane (7-TM) domains which are flanked by an extracellular N-terminus and an intracellular C-terminus (7). This structural motif is supported by a number of biophysical, biochemical and molecular biological studies on various GPCRs (8,9). In addition, recent molecular modelling studies based upon the primary amino acid sequences of a number of GPCRs have led to hypothetical three dimensional models which should be testable by experiment and may eventually lead to more rational approaches to drug design (10-13).

The recent literature (through early 1993) on the molecular and cell biology of select GPCRs in the CNS is summarized below. A discussion of all GPCRs in the CNS is beyond the scope of this review. Major emphasis has been placed on the molecular biology of dopamine and serotonin receptors since significant research activity has occurred in these two areas recently.

RECEPTOR CLONING STUDIES

Adenosine Receptors - Two subtypes of adenosine receptors (A_1 and A_2) have been characterized in the brains of various species based upon biochemical and pharmacological criteria. These receptor subtypes have been distinguished by their differential selectivity to adenosine agonists and antagonists as well as their opposite effects on intracellular cAMP accumulation: A_1 receptors inhibit adenylate cyclase while A_2 receptors stimulate it (14-17). In addition, a new subtype A_3, which couples to Ca^{++} metabolism, has also been suggested to be present in rat brain by pharmacological studies (18,19). The A_1 receptor has been cloned from a variety of tissues including the dog thyroid (20,21), rat brain (22), bovine brain and human brain (24). The human clone was obtained (326 amino acid residues) from a human hippocampus cDNA library by screening with a 2.3 kb CDNA probe from the dog A_1 receptor (RDC7). A clone exhibiting 96% similarity (94% identity) to the dog A_1 receptor was stably expressed in CHO cells. As expected for an A_1 receptor, it was found to inhibit the forskolin stimulated accumulation of cAMP after activation with the A_1 agonist N6-cyclopentyladenosine (CPA). In addition, the rank order of binding affinities for various adenosine agonists and antagonists was also consistent with an A_1 receptor (high affinity for the A_1 ligand ^3H DPCPX, no specific binding for the A_2 ligand ^3H CGS-21680).

The A_2 receptor has been described as having high affinity (A_{2a}) and low affinity (A_{2b}) subtypes (15). The orphan clone RDC8 (20) obtained from the dog thyroid cDNA library was recently characterized as the adenosine A_{2a} receptor. A human A_{2a} receptor was cloned from a human hippocampus cDNA library using the polymerase chain reaction (PCR) (25). Sequence analysis of the clone indicated that it encoded a 412 amino acid polypeptide which was similar (93% homology) to the dog thyroid A_{2a} receptor. When the pharmacology of the A_{2a} receptor expressed in human

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embryonic kidney 293 cells was examined it was found that the A_{2a} agonist ^{3}H CGS-21680 had high affinity while the A_1 receptor agonist ^{3}H CPA did not bind to the receptor. In addition, binding of ^{3}H CGS-21680 to stably transfected A_{2a} receptor led to the expected increase in cAMP production.

A clone characterized as the human A_{2b} receptor has also been reported (26). This human A_{2b} receptor contains 328 amino acid residues and when transfected into CHO cells showed significant production of cAMP when stimulated with adenosine. In addition, the A_1 selective ligand ^{3}H CPA and the A_{2a} selective ligand ^{3}H CGS-21680 did not bind specifically to the transfected receptor.

A novel receptor which has been designated as the A_3 adenosine receptor has recently been cloned from a rat brain cDNA library (27). The cDNA clone encodes a protein of 320 amino acids and exhibits 58% homology with the rat A_1 receptor (22) and 57% homology with the rat A_{2a} receptor (28). When the clone was expressed in COS-7 or CHO cells and characterized with adenosine agonists and antagonists, it exhibited a pharmacologic profile which was distinct from previously reported A_1, A_2 or A_3 receptors. The distribution of A_3 receptor mRNA in rat tissue was probed using reverse transcription PCR (RT-PCR). The highest expression was found in the testis with the kidneys, lungs and heart exhibiting moderate levels of mRNA. Low level expression was detected in the cortex, striatum and olfactory bulb. This contrasts to the distribution of adenosine A_1 and A_2 receptor mRNAs which are highly expressed in brain regions such as the cortex, hippocampus, cerebellum and thalamus.

Cannabinoid Receptors - Recent pharmacological studies now support the concept that cannabinoids produce their analgesic and psychoactive effects by a specific receptor mediated process rather than by nonspecific actions on cellular membranes (29). Further support for these observations has been provided by the cloning of the rat cannabinoid receptor from a cerebral cortex cDNA library (30). The clone was isolated using an oligonucleotide probe generated from a bovine tachykinin (NK_2) receptor and found to contain 473 amino acid residues. Interestingly, although cloned using a probe from the bovine NK_2 receptor, the cannabinoid receptor exhibited only 25% sequence homology. The receptor was stably expressed in Chinese hamster ovary (CHO) K1 cells and found to bind natural and synthetic cannabinoids with high affinity (potency ranking: CP 55940 > 11-OH-Δ^9-THC >(-)-Δ^9-THC > (-)-Δ^8-THC > cannabinol > cannabidiol). In addition, both Δ^9-THC and CP 55940 inhibited the forskolin stimulated accumulation of cAMP in a dose dependent manner as expected from tissue based pharmacologic studies. Using *in situ* hybridization techniques, the mRNA for this receptor was found in the hippocampus, cerebral cortex, ventromedial hypothalamic nucleus and the amygdaloid nuclei, brain regions where the cannabinoid receptor had been previously localized using autoradiographic studies (31).

The human homolog of the rat cannabinoid receptor was recently cloned from a human brain stem cDNA library using a low-stringency PCR method (32). A full-length clone was isolated and found to encode a protein of 472 amino acid residues which had 97% sequence homology to the rat cannabinoid receptor. The transmembrane regions of the rat and human receptors (where ligand binding is presumed to take place) were identical. When stably expressed in CHO-K1 cells, the human receptor exhibited a similar pharmacological profile to the rat homolog and to tissue preparations containing the cannabinoid receptor (30,31). In contrast to the rat cannabinoid receptor, the identical human receptor was also found in a human testis cDNA library.

Dopamine Receptors- Prior to 1990, two dopamine receptors (D_1 and D_2) had been characterized based upon pharmacological and biochemical criteria (33). Recent molecular biological studies have shown that considerable diversity exists in the dopamine receptor family: currently two D_1-like and three D_2-like genes have been identified by cloning studies (34,35). The rat and human D_1-like receptors (also described as D_{1A}) were cloned simultaneously in several laboratories (36-39) using low stringency screening of libraries or PCR using probes generated from the previously reported D_2 receptor (*vide infra*). Both the human and rat forms of the D_{1A} receptor contain 446 amino acid residues and have a high degree of sequence homology. The human D_{1A} receptor has been localized to chromosome 5 (38). When expressed in COS-7 cells, the pharmacology of the rat and human D_{1A} receptors was characterized as being similar to that described classically for D_1 receptors: high affinity for benzazepine D_1 ligands such as SCH 23390 and SK&F 38393; low affinity for D_2 ligands in the butyrophenone (spiperone and haloperidol) and benzamide (sulpiride) classes. As

expected, the cloned human D_{1A} receptor also coupled positively to adenylate cyclase when expressed in human 293 cells (37).

The highest levels of D_{1A} receptor mRNA in the CNS have been found in brain regions typically associated with dopaminergic function such as the caudate-putamen, nucleus accumbens and the olfactory tubercles. Lower levels of D_{1A} receptor mRNA were found in the cerebral cortex, hypothalamus, thalamus and limbic system. Interestingly, no D_{1A} mRNA was found in the substantia nigra, pars reticulata, globus pallidus and subthalamic nucleus - regions where high densities of D_1 binding sites have been described (36-39). This observation may be explained if the receptors are synthesized in the striatum and then transported to these regions as suggested by lesioning studies (40).

The second D1-like receptor was also cloned simultaneously by several groups and has been referred to as the D_{1B} (41), $D_{1\beta}$ (42) or the D_5 (43,44) dopamine receptor. Both the rat and human forms of this receptor have been cloned and found to exhibit considerable structural homology (95% identity in the transmembrane regions and 85% overall homology). The human D_5/D_{1B} receptor is located on chromosome 4. The D_5/D_{1B} receptor pharmacology was found to be similar to that observed for the D_{1A} subtype with the notable exception that dopamine had a 10-fold higher affinity for D_5/D_{1B} than for the D_{1A} receptor (44). In contrast to the D_{1A} receptor, the mRNA for the D_5/D_{1B} receptor has limited distribution in the rat CNS. The receptor mRNA does not appear to be present in the striatum, nucleus accumbens and olfactory tubercle (primary locations for the D_{1A} receptor). It is found in the thalamus and in several layers of the hippocampus (41).

During the cloning of the human D_5/D_{1B} receptor, several groups identified and characterized two pseudogenes present in the human genome (42,43,45). These pseudogenes were incapable of producing functional receptors due to the presence of defects (stop codons) in the nucleic acid sequence. These pseudogenes were detected in many areas of the human brain and may be capable of producing a peptide or truncated receptor containing 154 amino acids. It is unclear whether or not these pseudogenes play a biological role in regulating expression levels of the full length D_5/D_{1B} receptor (35).

The rat D_2 receptor was actually the first dopamine receptor to be cloned. It was isolated by low stringency screening of a rat brain cDNA library using the hamster β_2 receptor DNA as a probe (46). A human D_2 receptor was cloned from a pituitary cDNA library and found to have a protein sequence that is 96% identical to that of the cloned rat receptor with one notable difference: the human receptor contains an additional 29 amino acids in the third cytoplasmic loop (47). A second variant of the human D_2 receptor was detected in human caudate-putamen RNA using PCR (48). This receptor, similar to the initially characterized rat D_2 receptor, did not have the additional 29 amino acids that were found in the first human D_2 receptor. These alternatively-spliced variants of the D_2 receptor (long form: D_{2A}; short form: D_{2B}) have also been found in other species including rat, bovine, mouse and frog (35). In the human genome, both variants have been localized to chromosome 11 (35). Unlike the D_1-like genes, both D_{2A} and D_{2B} contain introns in their coding regions. Both forms of the D_2 receptor are expressed equally in the hippocampus and anterior cortex. However, the long form is present in higher abundance in the striatum. When expressed in CHO-K1 cells, both D_{2A} and D_{2B} exhibited similar dopamine D_2-like high affinity for spiperone and haloperidol as well as low affinity for SK&F 38393. While both receptor isoforms couple negatively to adenylate cyclase, the receptor subtypes are functionally distinct. At physiological dopamine concentrations, the D_{2B} mediated inhibition of calcitonin gene-related peptide stimulated cAMP accumulation is almost double the response mediated by the D_{2A} receptor. In addition, the D_{2B} form can maximally inhibit cAMP accumulation by up to 85% while the D_{2A} receptor is less effective, maximally inhibiting cAMP accumulation by only 64% (49). These observations highlight the potential importance of the tissue-specific or temporal expression of the subtypes. Variations in the strength of dopaminergic responses may play an important role in the generation of functional diversity in the CNS (49).

Previous biophysical and site-directed mutagenesis studies on the hamster β_2-adrenergic receptor have confirmed that a conserved aspartate residue in transmembrane helix 3 (TM3) was essential for epinephrine binding and signal transduction (8). The critical nature of this residue

(Asp^{80}) in the rat D_2 receptor has been recently confirmed by a site-directed mutagenesis study (50). As expected, replacement of Asp^{80} with Ala or Glu severely impairs inhibition of adenylate cyclase by the resulting mutant receptors. In addition, these substitutions abolish the Na^+ or H^+ regulation of the affinity of agonists and benzamide antagonists for the D_2 receptor. These data support the hypothesis that the conformation of D_2 receptors is maintained by interactions of monovalent cations with Asp^{80}. Additional site-directed mutagenesis experiments have also confirmed the importance of key residues Asp^{114}, Ser^{194} and Ser^{197} for agonist and antagonist binding (50).

The rat D_3 receptor was cloned by screening cDNA and genomic libraries using RT-PCR with a probe from the D_2 receptor (51). The rat gene encoded a protein containing 446 amino acid residues. When expressed in COS-7 cells, the cloned D_3 receptor was pharmacologically distinct from the cloned rat D_2 receptor: dopamine had 20-fold greater affinity to D_3 than D_2, the antagonists domperidone and haloperidol also had 30- and 20-fold, respectively, greater affinity for the D_3 receptor. The distribution of the D_3 receptor mRNA was studied using *in situ* hybridization and found to be similar to the D_2 subtype with several notable differences. The second messenger coupling of the D_3 receptor has not been characterized. When expressed in CHO cells, no coupling or high affinity binding was observed. Both D_3 and D_2 mRNAs were present in the olfactory tubule and nucleus accumbens. However, while D_2 was distributed throughout the striatum but was not found in the pituitary, D_3 was localized only in the ventral striatum and was found in the pituitary.

As was noted for the D_2 receptor, alternative splice variants for the D3 receptor have also been characterized in the rat (52), mouse (53) and human (54) genomes. Interestingly, the D_3 variants appear to be produced by a unique mechanism involving an internal acceptor site inside an exon rather than the existence of a separate cassette exon like in the D_2 variants (53). It is unclear whether or not the D_3 variants are functional receptors.

The human (55) and rat (56) homologs of the D_4 receptor were also cloned using probes generated from the D_2 receptor. The D_4 receptor exhibited very distinctive pharmacological properties. In general, most dopaminergic antagonists have much lower affinity for the D_4 receptor than for the D_2 or D_3 receptors. One notable exception is the atypical neuroleptic clozapine, which has an affinity at the D_4 receptor which is 6- and 20-fold greater than the its affinity at the D_2 and D_3 receptors, respectively. This has led to speculation that the D_4 receptor may be the relevant target for this neuroleptic drug (52). As for the D_3 receptor, the second-messenger coupling of the D_4 receptor was not characterized. *In situ* hybridization studies using the human clone in human brain showed the presence of D_4 mRNA in the caudate, putamen and the pars compacta of the substantial nigra. This distribution pattern was similar to the distribution for the D_2 clones in human brain. This is in contrast to the rat D_4 mRNA which is reported to be more widely distributed in the rat brain than the corresponding D_2 mRNA (57). Interestingly, at least three variants of the human D4 receptor have been reported (58). The presence of polymorphic receptors in the human population may underlie the differences in susceptibility to neuropsychiatric diseases as well as the responses to antipsychotic medications that are observed in patients.

Metabotropic Glutamate Receptors - Glutamate receptors are believed to play key roles in many neuronal functions in the CNS including neuronal plasticity, cognitive processes, memory and learning as well as some neurological disorders such as stroke. While the physiological importance of the ionotropic glutamate receptors (NMDA, AMPA, kainate and AP4) in the CNS has received considerable scrutiny, the role of the metabotropic glutamate receptor in CNS function is not as well characterized. This receptor differs from the ionotropic receptors in that it is a GPCR which activates phospholipase C (PLC) to produce IP_3 which releases intracellular Ca^{++}. The first metabotropic glutamate receptor ($mGluR_1$) was cloned from rat brain by two laboratories (59,60). The cDNA for this receptor encodes a protein of 1199 amino acids which contains the typical 7-TM domains which are flanked by very large amino- and carboxyl-terminal domains. This protein is considerably larger than other GPCRs. In addition, it exhibits no sequence homology with other GPCRs and very few conserved key residues. However, careful examination of sequence similarity with ionotropic glutamate receptors revealed that there is some sequence similarity between the amino termini of $mGluR_1$ and the AMPA/kainate receptors. When expressed in oocytes, $mGluR_1$ was activated by glutamate receptor ligands to release IP_3 with a profile that was typical for metabotropic glutamate receptors. RNA blot analysis showed that this receptor is distributed throughout different brain

regions and high levels of expression are present in the cerebellum and olfactory bulb. *In situ* hybridization revealed that $mGluR_1$ is expressed in many neuronal cell groups in the CNS including granule cells, CA4 cells in the hippocampal dentate gyrus, CA2-3 pyramidal cells and cerebellar Purkinje cells. Based upon this distribution, it has been suggested that $mGluR_1$ may be involved in evoking long-term potentiation (LTP) and long-term depression (LTD) (60). Three additional metabotropic glutamate receptors ($mGluR_2$, $mGluR_3$ and $mGluR_4$) have recently been cloned. These receptors show considerable sequence homology and similar distribution profiles in the CNS when compared to $mGluR_1$ (61).

Serotonin Receptors - During the past five years, pharmacologic and molecular biological studies have provided a wealth of information about the diversity and nature of serotonin (5-HT) receptors (62-65). In terms of diversity of CNS receptors, serotonin has no peers: far more 5-HT subtypes have been identified than any other CNS receptors of the GPCR class. This structural diversity has caused considerable confusion regarding the nomenclature of 5-HT receptors (66). Currently, each serotonin receptor subtype can be classified as being in one of seven 5-HT families: $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$, $5\text{-}HT_4$, $5\text{-}HT_5$, $5\text{-}HT_6$ and $5\text{-}HT_7$. Each family may be further subdivided into subfamilies: $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, etc.; $5\text{-}HT_{2A}$, $5\text{-}HT_{2B}$, etc. These families have been defined on the basis of receptor structure, receptor-effector coupling and pharmacology. As illustrated below, one must be quite careful when examining the recent serotonin literature because considerable evolution has taken place in the nomenclature of 5-HT receptors over the past couple of years.

The $5\text{-}HT_1$ serotonin receptor family is characterized by GPCRs which inhibit adenylate cyclase. The human receptor was the first $5\text{-}HT_{1A}$ receptor to be cloned (67,68) followed more recently by the rat species homolog (69). Transfection of the human $5\text{-}HT_{1A}$ clone into HeLa cells produces a receptor capable of both inhibiting adenylate cyclase activity (as seen in native brain tissues), and of weakly stimulating phosphoinositide hydrolysis, a property not described in native tissues (70). Originally the human 5-HT1A was localized only to lymphoid tissue (67). However, localization studies using the rat $5\text{-}HT_{1A}$ gene, identified the mRNA for this receptor in the septum, hippocampus, thalamus, amygdala, olfactory bulb, medulla, mesencephalon and hypothalmus, consistent with previous radioligand binding studies (69).

The rat $5\text{-}HT_{1B}$ receptor was cloned from a rat brain library (71) as well as from rat spleen (72). This receptor has long been considered a species variant of the human $5\text{-}HT_{1D}$ receptor. This was confirmed by comparison of the rat $5\text{-}HT_{1B}$ receptor with the cloned human $5\text{-}HT_{1D\beta}$ receptor (72,73). Comparison of the amino acid sequence of the rat gene with the human $5\text{-}HT_{1D\beta}$ gene showed 93% overall identity and 96% identity in the TM regions (72). Several other laboratories have also recently cloned the human $5\text{-}HT_{1D\beta}$ receptor (73,74) which they have described as the $5\text{-}HT_{1B}$ receptor. In a recent site-directed mutagenesis study, it was reported that mutation of a single residue in the binding region of the human $5\text{-}HT_{1B}$ ($5\text{-}HT_{1D\beta}$) receptor (from Thr^{355} to Asp^{355}), renders the pharmacology to be virtually identical to the rat $5\text{-}HT_{1B}$ receptor (75).

The $5\text{-}HT_{1C}$ receptor is considered to be in the $5\text{-}HT_2$ receptor family ($5\text{-}HT_{2C}$) and will be discussed below.

The orphan clone RDC4 obtained from a canine thyroid cDNA library was the first $5\text{-}HT_{1D}$ receptor to be cloned (76,77). Recently, a human $5\text{-}HT_{1D}$ gene, homologous to RDC4 has been isolated and characterized by several research groups (78-81). The human sequence of this receptor (termed the $5\text{-}HT_{1D\alpha}$ receptor) comprised 377 amino acid residues and exhibited 88% overall amino acid identity to the dog sequence, and 93% identity in the TM domains to the canine clone. As expected, this receptor also couples to the inhibition of adenylate cyclase. The binding affinities in the cloned $5\text{-}HT_{1D\alpha}$ receptor of a diverse set of 5-HT ligands correlated well with published affinities in human or bovine brain tissues (80). In situ hybridization studies indicate that the mRNA for this receptor is expressed in the pyramidal layer of the olfactory tubercle and the nucleus caudatus and accumbens (81).

As noted above, a second human clone from the $5\text{-}HT_{1D}$ family, termed $5\text{-}HT_{1D\beta}$, was recently isolated and characterized (80,83-85). This clone encoded a protein containing 390 amino acid residues which was structurally distinct from the $5\text{-}HT_{1D\alpha}$ receptor. The overall amino acid identity

between 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ was 63% with 77% amino acid identity in the TM regions. In addition, the pharmacological binding properties and second messenger coupling of 5-$HT_{1D\beta}$ were very similar to the properties exhibited by the 5-$HT_{1D\alpha}$ clone. *In situ* hybridization and northern blot analysis in human, monkey and rat tissues indicate that the 5-$HT_{1D\beta}$ gene is expressed in striatum, hippocampus and frontal cortex. In addition, this gene has been localized to human chromosome 6 (74).

The 5-HT_{1D} agonist sumatriptan has high affinity for both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ as well as 5-HT_{1F} (*vide infra*) receptors. Determining which 5-HT receptor mediates the anti-migraine effect of sumatriptan may allow for the identification of more efficacious agents for the treatment of this ailment.

Further heterogeneity of the pharmacologically defined 5-HT_1 receptors was suggested by radioligand binding studies showing biphasic competition curves for 5-carboxamidotryptamine (5-CT), ergotamine and sumatriptan. The low affinity binding component was termed either a "5-HT_{1D} subtype" or a 5-HT_{1E} receptor (86). The human and rat 5-HT_{1E} receptors have recently been cloned and characterized pharmacologically (86-88). The human 5-HT_{1E} clone encoded a protein containing 365 amino acid residues that had 64% identity with the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors and lower degrees of homology (35-52%) with other serotonergic or catecholaminergic receptors (88). When transfected into Y-1 cells, this receptor showed a binding profile in the presence of typical 5-HT receptor ligands that matched the profile from native brain preparations. In addition, the cloned receptor also showed the expected negative coupling to adenylate cyclase. The physiological role of the 5-HT_{1E} receptor has not been elucidated although it has been pharmacologically characterized in the brains of post-mortem suicide victims (89).

The human (90), mouse (91) and rat (92) homologs of the 5-HT_{1F} receptor have recently been cloned. These new clones represent the fifth receptor subtype in the 5-HT_1 family. The human gene encoded a protein 366 amino acids in length which had the following TM region identities with other 5-HT_1 receptors: 5-HT_{1E} (70%), 5-$HT_{1D\alpha}$ (63%), 5-$HT_{1D\beta}$ (60%) and 5-HT_{1A} (53%). When transfected in L-cells, competition studies using expressed 5-HT_{1E} receptor exhibited the following rank order of potencies for serotonergic ligands: 5-HT > sumatriptan > > 5-CT > 8-OH-DPAT > spiperone. When expressed in NIH 3T3 cells, the 5-HT_{1F} receptor was shown to be coupled to the inhibition of adenylate cyclase. The mRNA for this gene was detected in the human brain but not in kidney, liver, spleen, heart, pancreas or testes. The pharmacology and localization of the 5-HT_{1F} receptor do not resemble previously described 5-HT receptors. The elucidation of the physiological role in the CNS and the periphery will require further study and perhaps the identification of selective ligands.

The 5-HT_2 receptor family contains 3 receptors which are currently designated 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (66), each of which is coupled to phophoinositide hydrolysis (62). The human and rat 5-HT_{2A} receptors which were previously described as the "5-HT_2"subtype (66) have been cloned by several laboratories (93-97). The rat and human 5-HT_{2A} genes each encoded proteins of 471 amino acid residues. The overall sequence identity between the rat 5-HT_{1C} and rat 5-HT_2 receptors is 49%, but identity within the TM regions is 80% (94). The human and rat receptors have an overall sequence identity of 92%, but a 99% sequence identity in the TM regions (97). Of the 3 residues which differ in the human vs. rat 5-HT_{2A} receptors, the amino acid residue 242 on TM5 (Ser in the human receptor, Ala in the rat receptor) appeared to be most critical for ligand binding based upon previous studies on adrenergic receptors (8). When the Ser^{242} on the human receptor was mutated to Ala^{242}, the expressed mutant receptor displayed a 60-fold higher affinity for ^{3}H-mesulergine than the native human 5-HT_{2A} receptor. The affinity of the mutant receptor was very close to that observed for rat cortical membranes, a tissue preparation expressing 5-HT_2 receptors. This study suggests that Ser^{242} in the human receptor plays a critical role in ligand binding (97).

The 5-HT_{2A} receptor mRNA was found exclusively in CNS tissues: it was highly concentrated in the cerebral cortex and lower levels of expression were found in the hippocampus, hypothalamus, spinal cord and olfactory bulb (94).

The 5-HT_{2B} (previously named the 5-HT_{2F} receptor) was recently cloned from rat (98,99), and mouse (100) stomach fundus. The rat gene encodes a protein of 479 amino acid residues an shares greatest identity with the TM regions of the rat 5-HT_{1C} and 5-HT_{2A} receptors (70% and 68%,

respectively). This receptor was expressed mostly in the stomach, and heart with low level expression detected in the CNS (100).

The 5-HT_{2C} receptor (previously designated the 5-HT_{1C} receptor) was the first 5-HT receptor to be cloned (101,102). When expressed in NIH 3T3 cells, the 5-HT_{2C} receptor exhibited the expected pharmacologic binding properties and was capable of coupling to its appropriate second messenger system: pnosphoinositide hydrolysis. Subsequent *in situ* hybridization studies have shown that the receptor is located mainly on the choroid plexus with lower levels present in the hypothalamus, subthalamus and throughout the limbic system (103-105).

The mouse 5-HT_3 receptor was recently cloned (106). However, since this receptor is actually a ligand-gated ion channel rather than a GPCR, it will not be discussed here.

The 5-HT_4 receptor has been reported to positively couple to adenylate cyclase. Studies on this receptor have been hampered by the lack of a high-affinity radioligand (107). The 5-HT_4 receptor has been shown to have a wide tissue distribution in the CNS and periphery, and to mediate a number of functional responses. The cloning of the 5-HT_4 receptor has not been reported.

Two members of the 5-HT_5 family,the 5-HT_{5A} and 5-HT_{5B} receptors (also known as 5-$HT_{5\alpha}$ and 5-$HT_{5\beta}$, respectively) have been cloned from the mouse (108) and rat (109) brain. Their pharmacological profiles resemble the 5-HT_1 class of receptors based on their binding affinities for 5-CT and methiothepin. However, based upon the primary amino acid sequence, the receptors are intermediate in identity between the 5-HT_1 and 5-HT_2 receptors (109). It was not possible to demonstrate functional coupling of the 5-HT_5 receptors to either adenylate cyclase actibity or phosphoinositide metabolism. The 5-HT_{5A} receptor is widely distributed in the CNS, including the dorsal raphe nucleus. The 5-HT_{5B} receptor has a more limited distribution, although its mRNA was also localized to the dorsal raphe. Neither receptor was detected in peripheral tissues.

The 5-HT_6 receptor has also recently been cloned from rat brain (110). This receptor encodes a 437 residue protein which has approximately 40% identity within the TM regions when compared with 5-HT_2 and 5-HT_1 subtypes. Expression of this clone in COS-7 cells resulted in high affinity, saturable binding of ^{125}I-LSD. The pharmacological profile did not correlate with that of any previously described 5-HT receptor. In addition, this receptor exhibited high affinity for a number of antipsychotic and antidepressant drugs including clozapine, amoxipine and amitriptyline. When stably transfected in HEK-293 cells, the 5-HT_6 receptor positively coupled to adenylate cyclase.The mRNA for this receptor was predominantly expressed in the striatum, a region which is rich in dopamine receptors. It was also detected in the olfactory tubercle, cerebral cortex and hippocampus. The pharmacology and distribution of this receptor may indicate that it plays a role in mediating neuropsychiatric disorders.

Another novel 5-HT receptor (5-HT_7) which functionally couples to adenylate cyclase activation has recently been cloned (111). The human 5-HT_7 receptor was cloned from a fetal brain library. The mRNA for this receptor is expressed in human brain and a variety of human peripheral tissues. The amino acid sequence is reported to be significantly different from the 5-HT_6 receptor and the pharmacological profile diverges from that of the 5-HT_4 receptor, indicating significant complexity in the structure and pharmacology of 5-HT receptors which are positively coupled to adenylate cyclase.

CONCLUSIONS

Molecular biologists are clearly providing vast amounts of information about GPCRs at a very rapid pace. This review has focused on several key GPCRs which are under active study. It is worth noting that in addition to the receptors noted above, significant advances in the molecular biology of CNS neuropeptide receptors such as CCK/gastrin, neurokinin, neuropeptide Y and numerous other receptors are being reported at an accelerated pace (112). Information about receptor structure and diversity, tissue distribution and functional coupling of receptors to effectors is available because of the tremendous scientific advances that have occurred in the molecular biology of GPCRs. These techniques currently allow for the study of individual receptors in well-defined heterologous expression systems which are useful for the discovery of new therapeutic agents.

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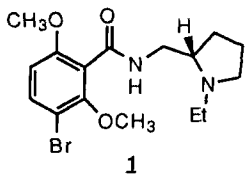
Chapter 5. Novel Antipsychotics

Harry R. Howard and Thomas F. Seeger
Central Research Division
Pfizer Inc
Groton, CT 06340

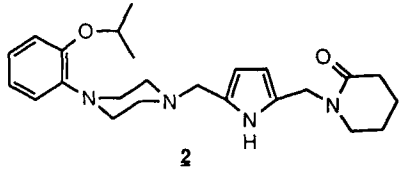
Introduction - The discovery of novel atypical antipsychotic agents with efficacy against both positive and negative symptoms, along with a reduced side effects profile, remains a primary goal in the treatment of schizophrenia (1-3). During the past year a number of reviews have appeared which cover emerging therapies (4-6) as well as the clinical performance of more advanced candidates including remoxipride (7-9) and risperidone (10-13). Additional studies have examined agents such as bromocryptine (14), fluvoxamine (15), mianserin (16), levopromazine (17) and zotepine (18), alone or as adjunctive therapies, for control of negative symptoms or for the treatment-resistant patient.

Dopamine Receptors - Further characterization of newly identified dopamine receptors (D_3, D_4, D_5) has included the first autoradiographic visualization of D_3 receptors in rat (19) and human brain (20). The D_4 receptor has been found to exist as several polymorphic variants, which may explain the genetic range of susceptibility to schizophrenia and / or varied individual response to antipsychotics (21). Agents with highly selective antagonist activity for any of the newly discovered dopamine receptors have not yet been described, although antagonism of D_4 receptors is of interest as a new target (22). There has also been considerable activity in the cloning and expression of dopamine receptors during the year, including work with the D_{1b} (23), D_2 (24, 25), D_3 (26) and human D_4 subtypes (27). Levels of mRNA for D_1, D_2, D_3 and D_5 receptors in rat brain after neuroleptic treatment have been measured (28, 29).

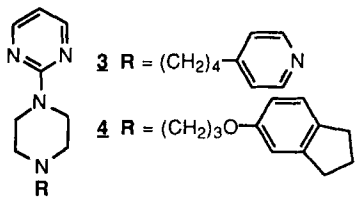
Dopamine (DA) Receptor Antagonists - In addition to remoxipride (**1**), a variety of D_2-receptor selective antagonists are reportedly under review. For example, RWJ-25730 (**2**) emerged as one of the most potent analogs from a series of disubstituted pyrroles (30). Two structurally similar pyrimidinylpiperazines **3** and **4** have been disclosed which show potent D_2 and 5-HT_{1a} antagonism and inhibition of locomotor activity in mice (31). A benzyl substituted analog (**5**) of spiperone with high D_2 affinity has been proposed as a potential PET tracer for in vivo D_2 receptor occupancy studies (32).



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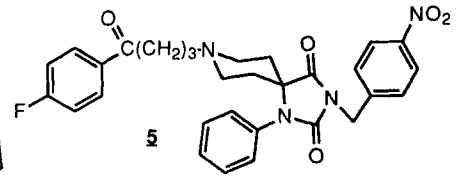


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3 R = $(CH_2)_4$-

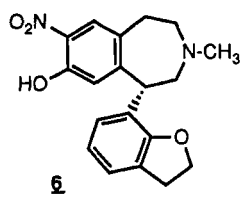
4 R = $(CH_2)_3O$-



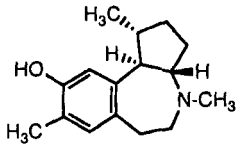
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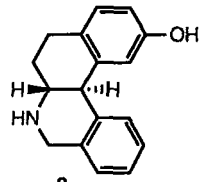
Antagonism of dopamine D_1 receptors has also been proposed as a method for the control of negative symptomatology in schizophrenia (33, 34). Among the more recently discovered D_1-selective antagonists are NNC-687 (6) and some compounds from a series of tricyclic derivatives, e.g., 7, which have proven active in the inhibition of conditioned avoidance response in squirrel monkeys (35, 36). A series of benzophenanthridines (e.g., 8) inhibit dopamine stimulation of adenylate cyclase activity and may possess cognition enhancement properties (37).



6

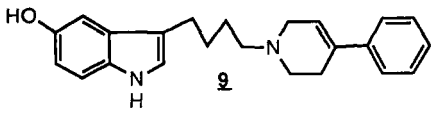


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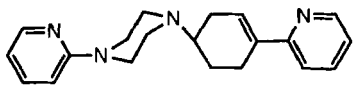


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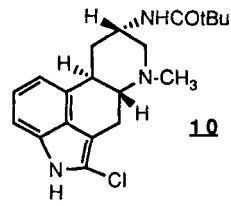
Dopamine (DA) Agonists - Clinical trials with roxindole (EMD-49,980, 9), a D_2 autoreceptor agonist, showed efficacy in a group of schizophrenic patients with primary negative symptoms (38, 39), whereas, results with SDZ-HDZ912 (10) have been less conclusive (40). Two autoreceptor agonists with selectivity for D_2 receptors - PD-135222 (11) and PD-138276 (12) - reduce amphetamine and apomorphine-induced locomotor activity in mice (41, 42).



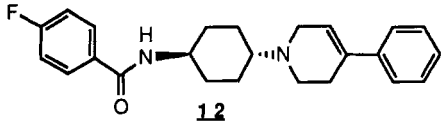
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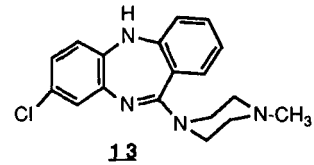
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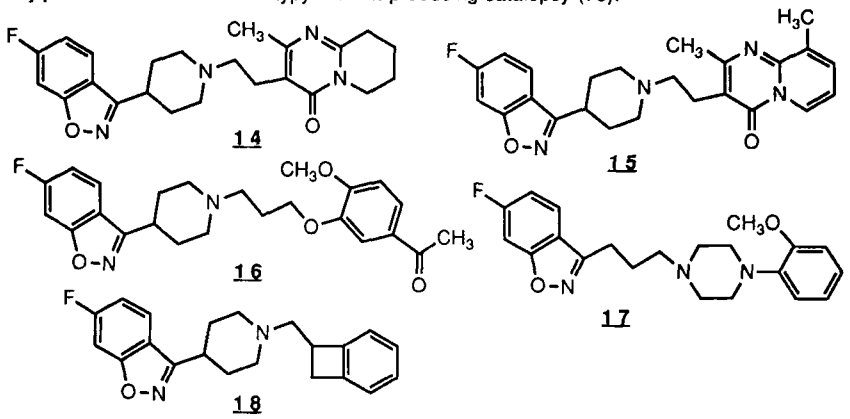


13

Clozapine - The virtues of this atypical antipsychotic continue to be extolled (43-46). Debate on the novel mechanism(s) of action of clozapine (13) continues; its superior efficacy has been attributed to interactions with a variety of receptor sites in the CNS, and this list has been expanded to include $5HT_{1C}$ (47), D_4 (48) and glutamate (49). The syntheses of ^3H- and ^{14}C-labelled 13 for mechanistic and metabolism studies have been described (50). A PET study using 13 showed that, within the efficacious dose range, a 36-52% D_1 receptor occupancy could be achieved versus a 38-63% level for D_2 sites (51), as compared to 70-90% occupancy seen with standard agents (52). The possible mechanisms underlying the development of agranulocytosis associated with 13 have been investigated. One proposal emphasizes a free-radical formation which may produce protein-adducts and subsequent induction of antibodies to these conjugates; the use of appropriate levels of ascorbate has been recommended for inhibition of free radical-mediated reactions. (53). Other similar immune response mechanisms have been proposed (54-57).

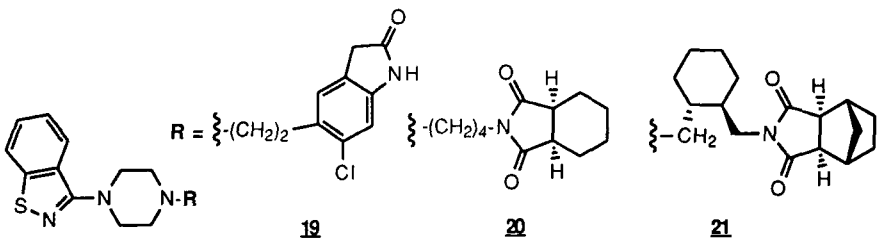
Serotonin (5-HT) Antagonists - The relevence of interactions of antipsychotic agents with serotoninergic receptors has been recently reviewed (58-60). In a 12-week double-blind study with treatment refractory subjects, patients receiving risperidone (14) required ten times less anti-

Parkinsonism medication than those on haloperidol (61); efficacy has also been demonstrated in patients with coexisting anxiety or depressive syndromes (62, 63). In vitro and in vivo, **14** is oxidized by cytochrome P4502D6 at the 9- and 7-positions of the tetrahydropyridopyrimidinone portion of the molecule (64). Ocaperidone (R 79,598, **15**) also combines potent 5-HT_2 and D_2 antagonism (65, 66); preclinical data suggest that **15** should produce less extrapyramidal side effects (EPS) (67). Other benzisoxazoles which display a similar preclinical profile are the piperidine HP-873 (**16**) which has entered Phase II clinical evaluation (68), the piperazine HRP-392 (**17**) which inhibited apomorphine-induced climbing in mice (69) and piperidine **18** which inhibited methylphenidate-induced stereotypy without producing catalepsy (70).

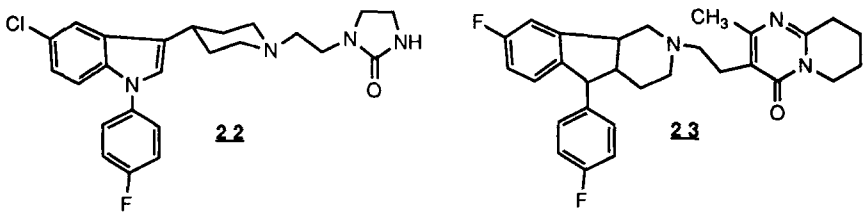


14 **15** **16** **17** **18**

The benzisothiazolylpiperazine moiety is also a common pharmacophore in several new combined D_2 / 5-HT_2 antagonists. For example, CP-88,059 (**19**), like SM-9018 (**20**), shows high affinity and selectivity for 5-HT_2 vs D_2 receptors in rat; at an oral dose of 40 mg **19**, PET studies determined a 70% D_2 occupancy in the putamen vs the D_2-selective antagonist [^{11}C]-raclopride (71). A series of analogs including **21** also bind with high affinity to the D_2 receptor and show little potential for side effects based upon the low level of catalepsy induction (72). The D_2 / 5-HT_2 selective antagonist, sertindole (**22**) has been evaluated in normal subjects for safety and tolerance (73). Finally, a series of indeno[1,2-c]pyridines, from which **23** was exemplified, combines selective D_2 and 5-HT_2 antagonism to give compounds which are active in the apomorphine-induced climbing assay (74).

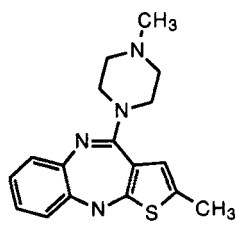


19 **20** **21**

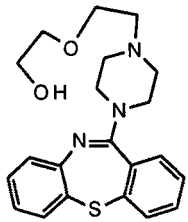


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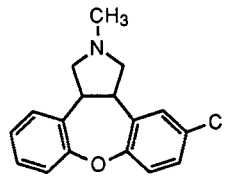
Structurally similar to 13, olanzepine (LY-170053, 24) combines D_2 and 5-HT_2 antagonism with high affinity for the D_1 receptor; it was tolerated in man with no evidence of agranulocytosis (75). The dibenzothiazepin ICI-204,636 (25), displaying affinity for both D_2 and 5-HT_2 receptors, has demonstrated efficacy in man with insomnia, sedation and transient sinus tachycardia being the major side effects reported (76, 77). Org-5222 (26) and Org-10490 (27) each exhibit potent antagonism of D_2 and 5-HT_2 receptors and are relatively free of EPS by comparison with other agents in recent clinical trials (73, 78).



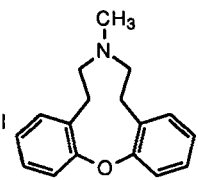
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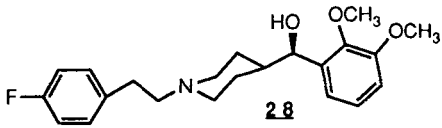


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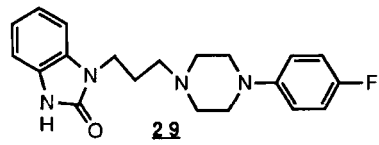


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Some investigators have continued to evaluate serotonin antagonists which possess little or no affinity for dopamine receptors as possible neuroleptics with minimal side effects potential. MDL-100,907 (28) is among the most 5-HT_2-selective antagonists, with weak affinity for D_2, 5-HT_{1a} and α_1 sites and with behavioral and electrophysiological activities consistent with that of an atypical antipsychotic agent (79). Also claimed to be selective 5-HT_2 antagonists are a number of benzimidazolones such as 29 (80).

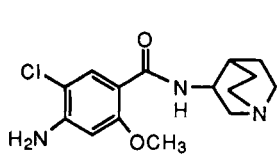


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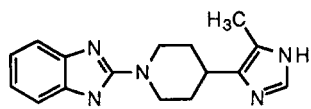


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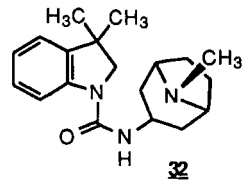
In a single-blind trial of neuroleptic efficacy with eight symptomatic patients, the 5-HT_3-selective antagonist zacopride (30) produced no significant improvements; almost all subjects experienced mild to moderate sedation and no significant changes in mean HVA or plasma prolactin levels were detected (81). Benzimidazolylpiperidines, such as 31, are claimed to be 5-HT_3 and/or 5-HT_4 antagonists with potential antipsychotic activity (82). BRL-46470A (32) has been mentioned as a possible antipsychotic agent with reduced EPS potential (83).



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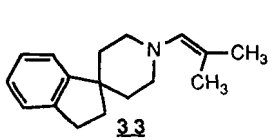
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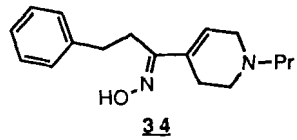
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Sigma - The potential role of sigma (σ) receptors in the etiology of psychosis has been discussed previously (see Chapter 1, this volume). Spiropiperidine analogs such as 33 have been claimed as among the most σ-selective compounds yet reported (84). The oxime PD-128,298 (34), is a selective σ antagonist which showed no EPS in haloperidol-sensitized monkeys (85), while DuP-734 (35) is selective for both σ and 5-HT_2 receptors, blocked mescaline-induced scratching and aggressive activity, and only weakly reversed APO-induced climbing, suggesting a low EPS potential (86-88). The novel σ antagonist XJ-887 (36), in addition to being a potent σ and 5-HT_2

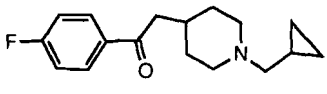
antagonist, displayed exceptional activity in the mescaline scratch assay and isolation-induced aggression model (89). Evaluation of a series of compounds related to the cyclohexane-diamine BD-737 (37, 90) demonstrated that enhanced potency and selectivity could be achieved through the greater conformational flexibility of acyclic diamines like 38 (91). From a series of aminoalkoxychromones, NPC-16377 (39) was found to reverse amphetamine-induced locomotion in mice (92).



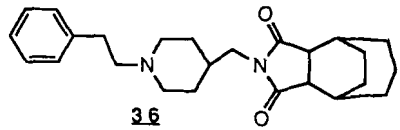
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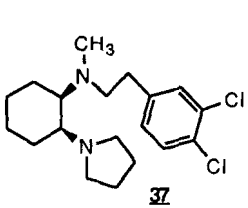
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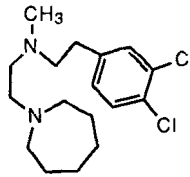
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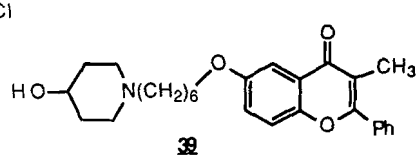
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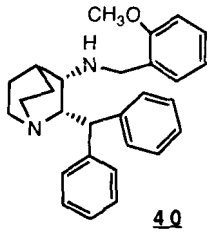


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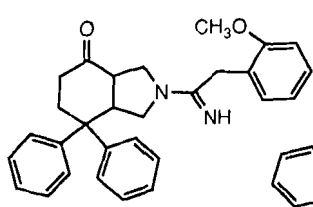


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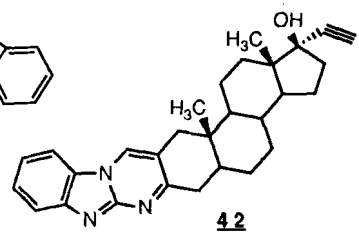
Neurokinin (NK_1) - Inhibition of neurokinin NK_1 receptors in the CNS has been been postulated as a means of regulating dopaminergic activity indirectly, thus minimizing the potential side effects associated with overt dopamine receptor blockade (93-95). The recent availability of selective, non-peptide antagonists such as CP-96,345 (40) has heightened interest in this approach; the SAR surrounding 40 has been published (96, 97) and a related ether series has also been disclosed (98). Other prototypical antagonists include the NK_1-selective amidine RP-67580 (41, 99-102) and a series of androstano[3,2-b]pyrimido[1,2-a]benzimidazoles from which WIN-51708 (42) was one of the most potent in vitro (103). Naphthimidazolium derivatives (e.g., 43) have demonstrated efficacy in a rat salivation assay (104). The human NK_1 receptor gene has been cloned and expressed from a human IM-9 lymphocyte cell library. (105).



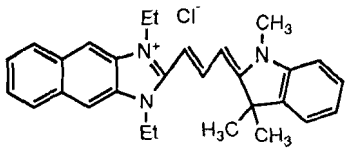
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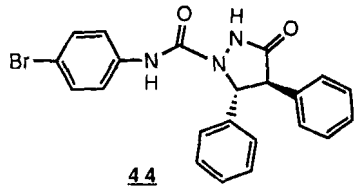
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Other Receptors - Several additional receptors have been proposed as having a potential role in the alleviation of psychotic symptoms. Cholecystokinin (CCK) receptors, upon exposure to the CCK-B selective antagonist LY-262691 (44), produced a reduction in the number of spontaneously active A9 and A10 dopamine neurons in the rat (106). An autoradiographic study has determined an alteration in the distribution of CCK receptors in schizophrenic brain (107). Regulation of D_2 receptors via adenosine A_2-selective agonists has been postulated as an alternative means of control (108, 109). Partial or full agonists for the glycine "co-agonist" site on the NMDA receptor may be useful in the treatment of schizophrenia without producing excitotoxicity (110) . Glycine agonists may, however, exacerbate symptoms for some patients, and their utility needs to be more fully investigated (111). Partial benzodiazepine agonists have also been proposed as yet another means of controlling psychotic behavior (112).

OTHER BIOLOGICAL FINDINGS

Etiology - The genetic factor in the development of schizophrenia has been further emphasized (113-116), as has the incidence of the disease due to prior exposure to viral pathogens. (117-120). The role of autoantibodies in the onset of psychosis has similarly been examined (121-124) and hypotheses related to imbalances in second messenger systems (125) and glutamate levels (126) have been advanced .

Mechanistic studies - A variety of studies have been directed at understanding the interaction of neuroleptics with CNS receptors utilizing PET (127-133) and MRI techniques (134-137). Molecular modelling studies, focused upon interactions between, for example, 5-HT_{1a}, 5-HT_2 and D_2 receptors and their corresponding ligands, have estimated the conformational changes which may be necessary for optimal antagonism (138-140).

Animal Models - A model of schizophrenia using a chronic methamphetamine intoxication protocol has been demonstrated (141).

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Chapter 6: Perspectives on Amyloid and Alzheimer's Disease: A Critical Review

Grant A. Krafft
Pharmaceutical Products Division
Abbott Laboratories
Abbott Park, Illinois 60064

Introduction - For decades, the role of amyloid in Alzheimer's disease (AD) has been debated. It is difficult to argue that there is not a connection between the two, since amyloid plaques are part of the neuropathologic definition of the disease first offered by Alzheimer (1), and remain the basis of current post-mortem AD confirmation (2). Nevertheless, it has been difficult to demonstrate definitively that amyloid or amyloid plaques are something significantly more than an epiphenomenon. This chapter summarizes recent studies that have sought to establish a clear, causal link between amyloid and AD neurodegeneration, and discusses these results within the hypothetical context of a neuronal "amyloid receptor". This chapter also highlights the tremendous challenge presented by a unique human disease that currently offers no view of early or ongoing pathology. Therapeutic approaches for AD are reviewed in Chapter 21 of this volume.

Background - The recent proliferation of molecular studies involving amyloid and AD was instigated by the pivotal studies of Glenner and Wong in 1984 (3) and of Masters and co-workers in 1985 (4,5), which established that ß-amyloid or ß/A4 amyloid (Aß), the major protein component present in AD neuritic plaques, was a 4 kDa, 39-43 amino acid peptide. Aß is also the major plaque component in Down's Syndrome patients (5). It is a poorly soluble peptide due to a hydrophobic C-terminal region and a central region that readily forms aggregates. Identification of the Aß peptide sequence led to cloning of the gene encoding the amyloid precursor protein (APP), an integral cell membrane protein from which Aß is derived (6,7). The APP gene is located on human chromosome 21 (8), and its transcripts can be alternatively spliced to produce APPs of 695, 714, 563, 751 and 770 amino acids (6,9). The latter three forms contain a Kunitz-type protease inhibitor domain, thought to be important in regulating extracellular serine protease activity (10,11).

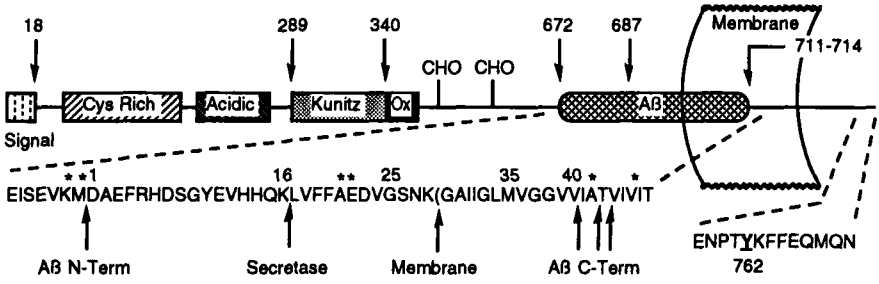


Figure 1. APP_{770} and the Aß sequence. Mutation sites designated by *.

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Transfection and expression of a cDNA encoding APP in HEK 293 cells established that APP could be proteolytically cleaved just beyond the transmembrane region, by an enzyme now known as APP secretase (12,13). This cleavage was shown to occur within the Aß peptide, thereby circumventing the formation of intact Aß. The prevalence or relevance of this secretory cleavage in the human brain is unknown, although recent studies of APP processing in differentiated P19 mouse embryonal carcinoma cells transfected with APP 695 cDNA, suggest that the secretory cleavage may be less prevalent in neuronal cells than in peripheral cell types (14). The discovery of this secretory cleavage led to early speculation that Aß is produced by an alternate, potentially aberrant proteolytic processing pathway, although recent studies indicate that Aß 1-40 is found under "normal" circumstances in cell culture (15,16), and also can be detected in serum and cerebrospinal fluid from normal adults (17,18). It also has been shown that a second secretase cleavage occurs at the N-terminus of Aß (19,20), enabling Aß generation by a subsequent C-terminal cleavage. Presently, the precise identity of the proteases that generate Aß from APP in cell culture or in human brain is not known, but this avenue of investigation is being intensively pursued. These studies will not be reviewed here, but it is worth noting that evidence variously supporting endosomal/lysosomal, extracellular, cytoplasmic, or cell surface venues for Aß formation has been obtained (21), and that neuronal receptor activation may influence Aß production (22). Whatever the mode of Aß formation, the key issue here is whether Aß, or APP fragments containing it are responsible for the selective neurodegeneration of AD.

THE ROLE OF AMYLOID IN ALZHEIMER'S DISEASE: GENETICS

The most compelling evidence supporting a causative link between amyloid and AD, is the existence of several different APP mutations linked to an inherited early onset form of AD (23-28). The first of these mutations was found in codon 717 of the APP_{770} gene, manifest as a valine to isoleucine replacement present in six early onset AD families (23-26). Further evidence that mutant APP may cause early onset AD emerged with the discovery of two different mutations, valine to glycine (27) and valine to phenylalanine (28), both also occurring at position 717 of APP_{770}. A fourth early onset AD-linked mutation, discovered recently in a Swedish family, consisted of a double replacement involving lysine to asparagine at residue 670, and methionine to leucine at the adjacent residue 671, positioned just upstream of the Aß N-terminal aspartic acid residue (29). Another well documented mutation associated with hereditary cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D), is a glutamic acid to glutamine mutation at position 693 (APP_{770}) within the Aß peptide (30). While these Dutch amyloidosis patients suffer from dementia, it was unclear whether the dementia was due to the cerebral hemorrhage or the accumulation and toxicity of amyloid. A recent report has described a 58 year old carrier of this mutation, who had not suffered cerebral hemorrhage, but who has shown progressive dementia from age 55, suggesting that Aß with the Dutch mutation also may cause AD-related dementia (31).

Although the mutations at 717, 670/671 and 692 of APP may well be causative of AD, they account for only a small percentage of the known familial AD (FAD) cases (32-34). Other studies have demonstrated linkage between FAD incidence and loci on chromosomes 14 (35-37) and 19 (38), which clearly indicate that AD is a heterogeneous syndrome rather than a single disease, in which pathways involving proteins other than APP may also lead to ultimate disease manifestations.

The linkage of APP mutations with AD provides strong evidence for a role of amyloid or APP in AD, but has not allowed elucidation of the mechanism by which the mutations exert their presumed pathogenic effects. Since all of the AD-linked APP mutations occur near the C- or N- termini of Aß, it is possible that the mutations alter the processing of APP to generate more Aß, or preferentially more Aß 1-42 which is less soluble and faster aggregating than Aß 1-40. It may be postulated that the mutations at position 717, which lie within the transmembrane domain of APP, destabilize the transmembrane helix, enabling higher levels of proteolytic cleavage at the Aß C-terminus. However, cell transfection studies incorporating APP with the valine to isoleucine mutation at 717 have failed to show an increase in Aß production (39,40). The secretory cleavage of APP also does not appear to be altered by this mutation (40). In the case of the Swedish double mutation, 6 or 8-fold higher levels of secreted Aß are produced in M17 human neuroblastoma cells (39) or HEK 293 cells (41), respectively, compared to wild-type APP transfectants, implicating higher levels of Aß production as the downstream pathogenic consequence of this mutation. The hypothesis that higher levels of Aß are pathogenic is supported by the close link between AD and Down's Syndrome, where APP levels are elevated, a circumstance which could lead to the substantially higher Aß levels found in Down's patients (42).

These genetic studies raise two possibilities regarding the involvement of APP in AD: i) that APP mutations result in altered APP metabolism to produce more amyloid, which may be neurotoxic, or ii) that APP mutations compromise normal, important functions of APP, and that compromised function eventually leads to neurodegeneration. To this point, distinguishing between these two possibilities has been difficult. However, as more information about the normal functions of APP emerges, these possibilities will be more easily evaluated.

BIOLOGICAL ACTIVITIES OF Aß

From the outset, studies of Aß biological activity and toxicity presented a puzzling and complex picture, not readily explained by a simple receptor-ligand model. The first study reported that Aß 1-40 and Aß 1-28 exhibited a neurotrophic effect, increasing the survival of cultured hippocampal neurons (43). A second study demonstrated that a recombinant 100 residue C-terminal APP fragment (C-100) containing the Aß sequence, expressed in cultured PC12 cells, caused these cells to degenerate gradually after differentiation into neuronal cells (44). Conditioned media from these cultures, apparently containing this C-100 fragment, was also toxic to primary hippocampal rat neurons, though media from cells similarly transfected with an Aß construct lacking the C-terminus of APP was not toxic. These studies suggested that at least two separate effects were occurring, presumably via two different pathways. The following sections suggest that the situation is even more complex than this.

In vitro Aß Peptide Studies - In 1990, Yankner and co-workers (45) were the first to attempt to reproduce the reported trophic and toxic activities of amyloid using synthetic Aß 1-40. Treatment of cultured hippocampal rat neurons with Aß 1-40 at low concentrations (1 nM) at the time of cell plating (day 0) elicited the trophic response, characterized by increased neuron survival in culture 1-3 days after plating. At higher concentrations (1-20 µM), Aß 1-40 was toxic, as characterized by lower survival of neurons 24 h later. Cells were more susceptible to Aß 1-40 the longer they had been in culture, a possible indication that Aß 1-40 was accelerating the normal death of cultured neurons, rather than mediating a direct, specific toxic effect. Evaluation of shorter peptides spanning the Aß sequence revealed that the

peptide fragment containing residues Aß 25-35 exhibited the same trophic and toxic activity as Aß 1-40, with the same dose-response relationship. However, the authors' suggestion that Aß 25-35 was responsible for all of the toxic and trophic effects of Aß, was somewhat premature in light of more recent studies (*vide infra*).

Aß 25-35 was noted to resemble several tachykinins, prompting experiments reported to show that substance P (SP) could block the toxic and trophic responses of Aß 1-40 when added simultaneously, although this was not the case for most other tachykinins (45). However, Aß 1-40 does not bind to the SP receptor (46,49), and the SP protective effect recently has been attributed to its ability to interfere with the formation of amyloid aggregates (47), although this would not explain how SP blocks the trophic response observed at concentrations well below the aggregating concentrations of Aß 1-40 (48). Other investigators also have failed to show any protective effect of SP (49-51).

The importance of the aggregation state of Aß 1-40 in defining its biological activity has been discussed by several investigators (51,52,54). The "aging" of Aß solutions, which enhances aggregation, increased *in vitro* toxicity, and also diminished the trophic or neurite promoting (52) activities observed at lower concentrations. Aß 1-40 at 10-20 µM does aggregate in cell culture after 1 day (53), so that the toxic effects observed by Yankner and co-workers may have resulted from aggregation of Aß while in culture. Studies with pre-aggregated Aß 1-42 (15 µM) demonstrated substantial toxicity and neuritic dystrophy (54,55). A control peptide from islet amyloid (IAP 20-29) that also aggregates readily, elicited no toxic response, indicating that the toxicity was not due simply to non-specific peptide aggregate interactions with the cultured neurons (54). A more thorough study from Cotman's laboratory indicated that aggregated Aß 25-35, 1-36, 1-39, 1-42, 1-47 and 1-52 all were toxic to mature, cultured cortical neurons at 25 µM, while non-aggregated preparations of these peptides were not toxic (51). Reduction of aggregation by addition of specific solvents also reduced the observed toxicity. The solvent used to administer amyloid *in vivo* also had a profound effect on toxicity, presumably by affecting the aggregation state (57). Another study reported that six different samples of Aß 1-40, chemically identical by sequence analysis, HPLC and mass spectrometry, but obtained from different sources, exhibited different toxicity (49). Only one of the six lots showed significant toxicity, as characterized by cell death 4 days after treatment of rat hippocampal neurons with 10 µM Aß 1-40 (49).

Soluble Aß 1-42 at 22 µM, which was not toxic by itself in one study (58), was shown to potentiate excitotoxicity elicited by glutamate, N-methyl-D-aspartate (NMDA) or kainate. At 20-80 µM, Aß 1-38 and Aß 25-35 destabilized calcium ion homeostasis, resulting in enhanced excitotoxicity by glutamate, NMDA or kainate (59). Aß 25-35 was more potent than Aß 1-38, and Aß 25-35 amide was equally effective as the free carboxylic acid. Below 10 µM, there was no enhanced excitotoxicity by any of the peptides. A similar enhancement of susceptibility to glucose deprivation, which induces excitotoxic damage, has also been attributed to soluble Aß 1-42 at 10 µM (60). Aß 25-35 at 10-100 µM directly increased intracellular calcium levels in NGF-differentiated PC12 cells, due to an influx of extracellular calcium (61). This effect was dose-dependent, and was not observed for Aß 1-28 or Aß 12-28. Whether excitotoxic damage enhanced by Aß plays a role in AD is not known, although some tangle pathology similar to that seen in AD has been reported in instances where excitotoxic damage has occurred (62). Aß 1-40 has the ability to form calcium channels in bilayer membranes, resulting in an influx of calcium. The effect is cation specific, though in the presence of calcium, the bilayer membranes also become permeable to various monovalent metal ions (63).

Aß 25-35 and Aß 1-42 are directly toxic at relatively low concentrations (EC_{50} =7 or 20 nM, respectively) in cultured PC12 cells differentiated with nerve growth factor (64). Surprisingly, this effect could be blocked by vitamin E (100 µg/mL) even at Aß concentrations as high as 10 µM (64), an effect that the authors related to its antioxidant properties, since a similar protective effect was observed with the antioxidant, propyl gallate. Several other cell types suffer some toxicity to Aß, including Swiss 3T3 fibroblasts which suffered 50% cell death from 10 µM Aß 25-35 (64). This effect could well be caused by non-specific effects, such as the calcium channel effect described above (63).

Taken together, the *in vitro* studies summarized above suggest that Aß and certain shorter peptides are toxic to neurons in the published paradigms. It appears that aggregation may be required for some, if not all toxic manifestations in this type of *in vitro* study, which may explain some of the variable toxicity results that have been obtained. However, it is probable that some Aß effects also may be mediated by the soluble, monodisperse peptide. The nanomolar toxicity of Aß 25-35 and Aß 1-42 on PC12 cells does suggest a specific, receptor mediated activity, yet all of the other toxic effects observed in neuronal cell culture systems require relatively high concentrations of Aß, suggesting that specific receptor mediation may not be involved, and raising concerns regarding the relevance of these *in vitro* effects to the neurodegeneration and cell death that occur in AD. In a majority of studies, the primary parameter by which Aß effects have been characterized is simply cell death, a measure that does not permit assessment of some of the very specific responses of neurons that occur in AD, such as the phosphorylation of the tau protein which can lead to the formation of neurofibrillary tangles (65).

Toxicity of Transfected APP Constructs - The toxicity of recombinant C-100 and APP has been studied by three laboratories. PC12 cells expressing the C-100 fragment slowly degenerated over a period of 7 days following differentiation with nerve growth factor (*vide supra*) (44). C-100 transfected NIH 3T3 fibroblasts were not affected, nor were undifferentiated PC12 cells, indicating specificity for the neuron-like phenotype. A surprising observation was the toxicity induced by conditioned media from C-100 transfected PC12 cells or NIH 3T3 cells when administered to untransfected, NGF treated PC12 cells or cultured primary rat hippocampal neurons. The toxicity of the media was diminished by immunoadsorption with either of two anti-C-100 antibodies, suggesting that the C-100 fragment had been released into the media, and was responsible for the toxicity. Conditioned media from cells similarly transfected with a construct encoding only Aß, or one encoding full length APP was not toxic (44).

The biological activity of the APP C-terminal domain was demonstrated more precisely in a recent study (68) that showed specific binding (Kd = 0.8 nM) of the C-100 fragment to a recognition site on the surface of PC12 cells. Both the binding and the toxicity were shown to have an unusual pH dependence, with toxicity and binding falling off dramatically above pH 7.8. Expression of an analogous C-100 protein containing a tyrosine to phenylalanine replacement at residue 762 (APP_{770}), nine residues from the C-terminus, was not toxic to the NGF treated PC12 cells. The phenylalanine mutant protein did not interfere with the binding of the native sequence (Kd > 10 µM), indicating that the tyrosine was crucial for binding activity. It was suggested that phosphorylation of this tyrosine may facilitate binding and attendant toxicity (68). This demonstration of specific binding does suggest that an "amyloid receptor" may mediate toxicity in PC12 cells, yet it is clear that this receptor requires more that the 40 or 42 residues of Aß for its ligand. It is also clear that this "amyloid receptor" is different from any putative receptor that mediates the activities of Aß peptides described above.

Expression of the C-100 fragment in P19 cells, a cell line that differentiates into mixed populations of neurons and astrocytes when treated with retinoic acid (RA), resulted in stable transfectants of undifferentiated cells (69). However, substantial cell death occurred several days after RA-induced differentiation. Four C-terminal APP fragments were produced by the C-100 transfectants, 16 kD, 14 kD, 13 kD and 11 kD. Only the two smaller fragments appeared in the non-transfected P19 cells, which upregulated endogenous APP synthesis upon RA treatment (14). Among several distinct P19 clones expressing the C-100 protein, the time course and extent of degeneration was variable, and appeared to coincide with the levels of the 16 kD and 14 kD bands produced by these clones.

In a separate study (70), P19 cells stably transfected with APP_{695} or APP_{770} cDNA, showed substantially diminished populations of neurons 3-5 days after differentiation with RA. Undifferentiated or non-neuronal cells did not degenerate. By day 8, no neurons remained in these cultures. Western blot analysis of APP-derived fragments in the degenerating neuronal cultures using a C-terminal anti-APP antibody revealed many heavy protein bands, including a prevalent 12 kD band, and many fragments in the 16-35 kD range, all long enough to contain the full length Aß. Most of these amyloidogenic APP fragments were membrane bound. Ustilizing the human APP promoter to drive C-100 expression, no surviving neuronal cells were produced in cultures of RA differentiated P19 cells (71). Only proliferating non-neuronal astrocyte progenitor clones were isolated. Yoshikawa also has not been able to obtain P19-derived neurons expressing C-100 (72).

These studies from three different laboratories implicate the C-100 fragment, or comparable amyloidogenic fragments derived from APP, as mediators of toxicity that is specific to neuron-like cells (68-72), specificity which may derive from neuron-specific expression of "amyloid receptors". The differences in toxicity for full length APP observed in these studies may be attributable to different levels of expression. Lower overexpression of APP (43) may be tolerated by neuronal cells, presumably because the toxic amyloidogenic C-100-like fragments are not produced at sufficiently high levels. Higher overexpression may force substantial APP processing by a pathway that generates higher levels of the amyloidogenic fragments (70). High expression of C-100 may kill off neuronal cells shortly after differentiation (70,71), while lower levels of C-100 may enable neuronal survival for several days (69), with eventual degeneration and death. Evidence that tyrosine 762 (APP_{770}) near the C-terminus plays a crucial role in binding and toxicity in PC12 cells indicates a requirement for the C-terminal domain of APP. Why full length APP was not toxic in the early study (43) is unclear, since the C-terminal domain is obviously present. It is possible that in full length APP, tyrosine 762 does not undergo phosphorylation as speculated for the recombinant C-100 fragment (68), or that the C-terminal domain is never released from cells to interact with the PC12 cell surface binding site. Establishing the relevance of these *in vitro* PC12 studies to AD neurodegeneration will require identification of this binding site as a neuron-specific receptor that exists on certain human neurons.

In vivo Amyloid Studies - The quest for a relevant animal model for AD began with experiments involving direct injection of Aß into rat brains. The first report by Kowall and co-workers described significant lesioning and neurotoxicity of solubilized Aß 1-40 injected locally in rat cerebral cortex and hippocampus (73). Co-injection of SP antagonized these neurotoxic effects (73). Direct injection of Aß-containing plaque cores from AD brain caused significant neurotoxic effects in 7 of 10 rats, and these effects were observed after one month, but not within two or seven days following injection (74). Neurons in direct proximity to the injected cores suffered the greatest loss, and exhibited some staining with Alz50, an antibody with

modest selectivity for tangle pathology in AD (75). A significant inflammatory response was elicited by injection of the plaque cores, which may have contributed substantially to the pathology observed (75). This is consistent with observations of inflammatory and immune phenomena associated with AD (76), and with recent *in vitro* studies demonstrating that Aß 1-40 can activate the complement pathway by interaction with the complement component C1q (77, 78).

Since these initial reports, the effects of injected Aß *in vivo* have been studied extensively (79-88). Three groups of investigators have reported neurotoxicity of Aß peptides (79-81). In one study, human Aß 1-40 and rat Aß 1-42 (3 different residues) were injected into the hippocampus, dissolved in three different solvents (water, 35% acetonitrile, 30% cyclodextrin/PBS), with the reversed peptide Aß 40-1 used as a control (80). With cyclodextrin, minimal toxicity was observed, but with 35% acetonitrile, the human Aß 1-40 and rat 1-42 were consistently more toxic than Aß 40-1. With water as solvent the difference in toxicity was not as great. SP did not protect in this study, as had been reported previously (73). Another study confirmed similar toxicity of injected Aß 1-40 in rat brain (79), and neurotoxicity was documented for injected Aß 1-40 and Aß 25-35 in both rat and monkey cerebral cortex (81). The monkey pathology was described as more closely resembling neuronal degeneration in AD, on the basis of Alz50 staining somewhat characteristic of AD tissue. Another study evaluated Aß 1-40 and Aß 25-35 in similar rat brain injections, but the authors of this study suggested that physical aggregation and tissue displacement were responsible for the neuronal and tissue loss that occurred, rather than a specific neurotoxic effect (82).

Six studies have reported unsuccessful attempts to induce significant pathology in rat brain (83-86) or monkey brain (87,88) by Aß injections. Some damage was observed, but in comparison with control peptides or solvent, the damage was not considered significant, or characteristic of authentic AD pathology. The monkey brain injections of Aß 1-40, Aß 25-35, Aß 40-1, and scrambled Aß 25-35 all produced small lesions after two weeks or three months, and staining showed reactive astrocytes and activated microglia immediately surrounding the injection tracts (87,88). A few dystrophic neurites were observed around regions that immunostained with anti-Aß antibody, but there was no significant difference between controls or Aß peptides. Silver staining or immunostaining with Alz50 or the anti-tau monoclonal 5E2 showed no AD or AD-like pathology (87,88).

The extensive efforts to establish Aß peptide injection as a viable model for AD, or to establish amyloid toxicity as the cause of AD, have not permitted solid conclusions to be drawn. This is not to discount those reports describing pathology that may share some characteristics with AD pathology, but rather to emphasize that studies must be reproducible and quantitative with respect to disease-relevant mechanistic parameters if they are intended to serve as models to evaluate new therapeutic strategies or measure the efficacy of AD drug candidates.

Transgenic Animal Models - A complementary approach to obtain animal models for AD and to assess mechanistic hypotheses is the production of transgenic animals. The first report of a transgenic mouse line described the accumulation of Aß immunoreactivity in the dendrites of some but not all hippocampal neurons of 1-year-old mice expressing Aß 1-42 under control of the human APP promoter (89). Aggregates of Aß were observed to form amyloid-like fibrils similar to those appearing in AD in these mice. Unfortunately, subsequent examination of aged control mice from this C57BL/6J strain indicated that similar deposits were present (90), calling into question the conclusions of the original paper (91).

Transgenic expression of the C-100 fragment under control of the a viral promoter was achieved selectively in glial cells of five mouse lines, and more Aß immunoreactivity was observed in transgenics than controls, but no pathology was reported in these animals (92). Similarly, expression of APP_{695} was achieved in transgenic mice, which were reported to have impaired spatial memory, yet no pathology in these mice was reported (93). Transgenic mice overexpressing APP_{751} with the neuron specific enolase promoter have exhibited amyloid deposits in cortical and hippocampal brain regions, while similar transgenics expressing APP_{695} lacked these deposits (94), suggesting that overexpression of the Kunitz containing isoform may be a pathogenic event. The most encouraging report of AD transgenic mice described pathology virtually identical to human AD, including dense core plaques and neurofibrillary tangles (95), but this report was retracted due to failure to reproduce any of the originally reported pathology (96).

Overexpression of the C-100 fragment under control of the brain dystrophin promoter was achieved in 12 transgenic mouse lines, with transgene copy number greater than 20 in several lines (97). All of the transgenic animals analyzed showed abnormal intraneuronal amyloid deposits, some of which appeared similar to some deposits in AD brain. Thioflavin S fluorescence staining in mice with the highest expression levels also was observed in and around cerebral blood vessels, indicating the presence of Aß. There was no report of Alz50 staining, neurofibrillary tangle pathology or cytoskeletal abnormalities similar to AD pathology in this paper.

None of the valid transgenic studies documents neurodegeneration, and none represents even a partial model of AD pathology. Technologic successes have been achieved in brain specific expression of APP or amyloid fragment transgenes, which may be important for future success. Many efforts to obtain transgenic animal models of AD currently are in progress.

SUMMARY AND FUTURE PROSPECTS

Is amyloid toxic? Yes -- to neurons in culture and to brain tissue in animals when injected under certain circumstances. Is amyloid toxicity responsible for AD? Perhaps yes, but the work reviewed in this chapter does not provide definitive support for this. What are the appropriate experiments that will definitively implicate amyloid? For *in vitro* studies, the endpoints to assess amyloid activity must extend beyond counts of cell death, to include measures of specific cellular responses that initiate and mediate the progression of normal neurons to degenerated, tangle-filled neurons. These responses then must be correlated with events or responses that have occurred in cells from AD brain. Thus, it is important to continue searching for AD-specific molecular markers within brain tissue, and specifically within AD neurons, in order to validate responses that can be induced and measured in cultured human cells *in vitro*. If results continue to suggest that amyloid is involved in AD, then they must also explain the specific neuronal susceptibility manifest in selected parts of the brain to the effects of amyloid, which is deposited throughout the AD brain. For *in vivo* studies, rodents may not be appropriate models, since no study has shown definitively that rodent neurons degenerate in a manner at all similar to the neurogenerative process that occurs in AD. Since rodent and human Aß are different at three of the first fifteen amino acid residues, human Aß may not interact with specific rodent neuronal "amyloid receptors" in the same way that it may interact with human receptors. The ultimate proof of mechanism could emerge from the appropriate transgenic animal model, but it is important to understand that AD ultimately may be a disease that specifically affects humans because of the unique structures of particular human proteins (e.g APP), the unique functions of certain human neurons and the unique characteristics of human brain architecture.

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Chapter 7. Non-peptide Ligands for Neuropeptide Receptors

David C. Rees*
Parke-Davis Neuroscience Research Centre
Addenbrookes Hospital Site, Hills Road, Cambridge CB2 2QB, U.K.

(*Current Address: Organon Laboratories, Newhouse, Lanarkshire ML1 5SH, U.K.)

Introduction - The growing interest in the discovery of non-peptide ligands which bind to receptors for endogenous peptides is reflected by the number of publications which now use the words "peptoid", "peptidomimetic" or "non-peptide" to attract the attention of bioorganic, synthetic organic and medicinal chemists alike (1). The reason that non-peptide ligands are sought resides mainly in the well documented limitations of peptides as drugs; i.e. poor oral bioavailability, poor in vivo half life and poor CNS penetration. The historical development of this field can be traced back to the discovery of the endogenous opioid peptides, e.g. Met-enkephalin : Tyr-Gly-Gly-Phe-Met (2). The non- peptide opioid alkaloid drugs, e.g. morphine (1), are the prototype "non-peptide ligands for neuropeptide receptors". Although this chapter is concerned with neuropeptides the important contributions that have been made to the discovery of non-peptide enzyme inhibitors are noted (3).

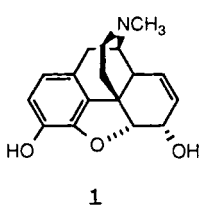
Strategies for the Discovery of Non-peptide Ligands - Sufficient work has now been published in this area to review not only the structures of non-peptide ligands but also the strategies which have led to their discovery. In this regard, the pioneering conceptual contribution of Ariëns and Farmer (4) deserves special mention. Based on thermodynamic grounds they proposed that it should be possible to achieve nanomolar receptor binding affinity with (only) three appropriately oriented binding groups. For a peptide receptor these binding groups can be selected from the amino acid side-chains. This implies that it is not necessary to retain the full peptide structure in order to achieve high binding affinity. Furthermore, they proposed various organic "templates" onto which these binding groups could be appended. Several subsequent publications which have utilized these concepts are discussed below. More recently, Hirschmann and co-workers have summarised three distinct approaches for identifying non-peptide ligands : i) broad screening, ii) the design and synthesis of peptide analogs possessing amide replacements and iii) the design and synthesis of novel scaffolding with retention of peptide side-chains (5). The broad screening approach, which includes natural product sources and company files, has been facilitated by the automation of high throughput receptor binding assays. Many of the non-peptide ligands in this chapter are reported to have been discovered with the aid of a broad screening approach. The other two strategies incorporate an element of design based on the structure and the peptide-SAR data of a known peptide antagonist or an endogenous peptide agonist. Examples which illustrate these different strategies are given in this chapter. The vast majority of non-peptide ligands of neuropeptide receptors are antagonists. The best example where clear agonist efficacy has been demonstrated comes from the opioid field.

Cholecystokinin - It is appropriate for cholecystokinin (CCK) to be the first neuropeptide discussed in this chapter because all of the strategies mentioned above have been used to identify non-peptide ligands for the CCK-A or CCK-B receptor subtypes.

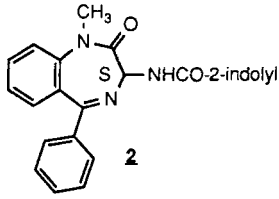
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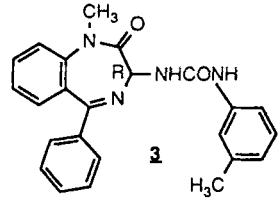
Broad screening furnished asperlicin, a natural product non-peptide lead with modest affinity for the CCK-A receptor. This provided the starting point for the development of high affinity, selective CCK-A antagonists such as devazepide (MK 329, 2) and CCK-B antagonists such as (L-365,260, 3) (reviewed in refs. 6,7). An independent chemical strategy also starting with asperlicin led to a series of quinazolinone CCK-B ligands exemplified by (4) (see table 1) (8,9). Elsewhere, another strategy of broad screening followed by lead optimisation led to the diphenylpyrazolidinone series of CCK-B antagonists typified by LY 262691, (5) (10). Two of these apparently unrelated CCK-B antagonists (3 and an enantiomer of 5) have been compared in a molecular modelling overlay study. This reveals that the spatial orientation of the three aromatic rings and the urea group for each compound may be very similar. It has therefore been suggested that the pyrazolidinone and diazepine rings function as somewhat rigid templates to orient the key binding fragments (10).



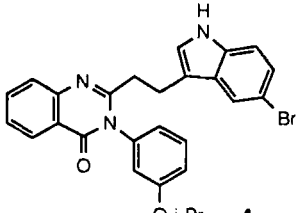
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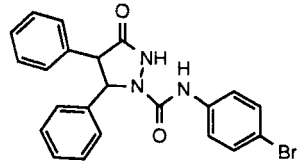
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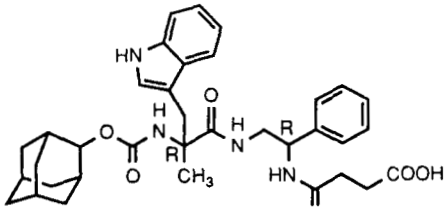
5 (trans)

The strategy leading to the discovery of the above CCK-antagonists relies heavily on broad screening. However, medicinal chemists have also designed non-peptide, selective CCK antagonists starting with the structure of the endogenous peptide (reviewed in ref. 11). This strategy is summarized in fig. 1. The key steps include : the identification of CCK (30-33) as the minimum fragment of the endogenous peptide CCK (26-33); the identification of a weakly active non-contiguous dipeptide; the incorporation of a methyl group at the α-C of the Trp residue, and subsequent N- and C-terminal modifications leading to CI-988 (PD 134308, 6).

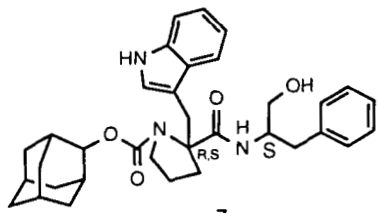
Figure 1 : Summary of Development of CI-988, 6 (11).

Compound	CCK-B receptor binding affinity (K_i)*
Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (CCK 26-33)	3 nM
↓	
Trp-Met-Asp-Phe-NH_2 (CCK 30-33)	3 nM
↓	
Boc-Trp-Phe-NH_2	70 μM
↓	
Boc-αMeTrp-Phe-NH_2	6 μM
↓	
CI-988, 6	1.7 nM

*These binding data are obtained in the same laboratory.



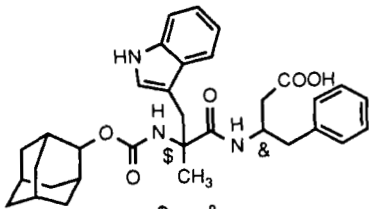
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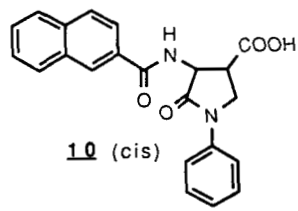
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The pronounced effect of α-methylation of Trp is ascribed to a 300-fold difference in conformational entropy between the α-Me and α-H compounds, which is explained by the conformational restriction of four C-C bonds (12). Further conformational restriction based on the desirable effects of this α-Me group led to the Pro derivative (7). This compound was indicated as a suitable conformationally restricted derivative based on molecular dynamics simulations of the amide backbone and also based on the X-ray crystallographic conformation of CI-988 in which the C-α-Me and carbamate N-H bonds are eclipsed (13). The COOH group of CI-988 has been replaced with a variety of acid mimetics which span a pKa range of < 1 through 9.5 (14).

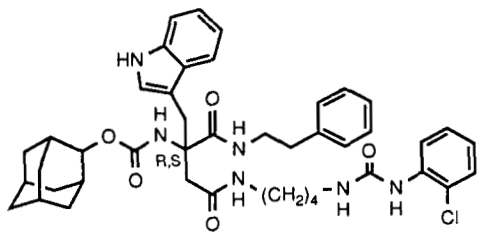
In an interesting development to the CI-988 story, it has been shown that changing the absolute stereochemistry of both chiral C-atoms in the CCK-B selective ligand PD 135666 (8) produces a CCK-A selective ligand (PD 140548, 9) (15).



	$	&
8	R	S
9	S	R



10 (cis)



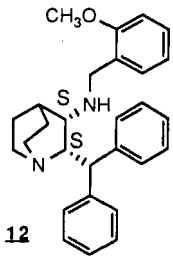
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Another design strategy mentioned above involves the construction of a template (or scaffold) onto which key binding groups (e.g., amino acid side-chains) are appended. This concept has been reduced to practice by the discovery of the CCK-A antagonist SC-50998, (10). Here the template is a 1,3,4-trisubstituted pyrrolidinone, in order to achieve CCK activity mimetics of the amino acid side-chains of Trp^{30}, Asp^{32} and Phe^{33} of CCK (27-33) were appended. SC-50998 is selective for the CCK-A receptor and behaves as an antagonist in vitro and in vivo (16). Compound 11 is a CCK-A ligand which is reported to utilize a

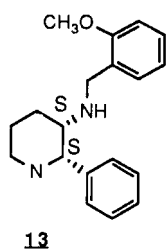
single tetrasubstituted sp^3 carbon atom as a template (17). This has structural similarity to the CCK-B antagonist CI-988 but the key difference is the substituted Lys side-chain appended to the α-Me carbon on the Trp residue. This same moiety has been shown elsewhere to confer CCK-A activity to some CCK (30-33) pseudopeptides (7).

The final strategy discussed here involves the replacement of metabolically labile peptide amide bonds. This "amide replacement" approach has been very successful in designing enzyme inhibitors, but it has generally not enjoyed the same success when applied to neuropeptide ligands. For example, the central amide bond within a series of CI-988 derivatives has been replaced with eleven isosteres but all of these led to significantly weaker CCK-B receptor binding affinity (18). However, it has been shown that the CCK-A receptor binding affinity of the tetrapeptide Boc-Trp-Lys(hyc)-Asp-Phe-NH_2 (hyc = 4-hydroxycinnamoyl) is retained when the Lys(hyc)-Asp peptide bond is replaced with a methylene-amino isostere (19).

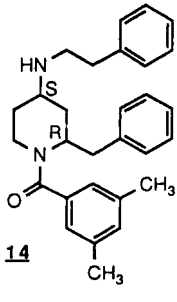
The discovery of non-peptide ligands with selectivity for the CCK receptor subtypes has been accompanied by an active investigation of their pharmacological properties (6,7,11). It now appears that the obesity/appetite indications are associated with the CCK-A receptor (20) while the anxiety/panic effects are mediated by the brain CCK-B receptor (21). In addition, it has been shown that CI-988 dose-dependently antagonizes both the anxiogenesis and the proconvulsant effect following diazepam withdrawal in mice. This, together with the observation that CCK co-exists with dopamine and GABA, has led to the speculation that this class of compounds may have therapeutic utility in treating substance abuse (22,23).



12



13

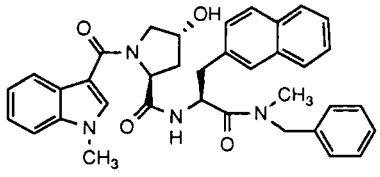


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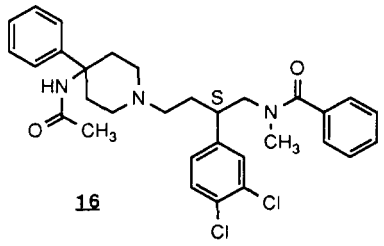
Neurokinin-1 - A breakthrough in the tachykinin area came in 1991 with a report that a chemical file-screening approach led to the quinuclidine, CP-96345, (12) which is a potent NK_1 receptor antagonist (NK_1 receptor binding in bovine caudate nucleus, K_i = 0.6 nM, pA_2 = 8.7 isolated dog carotid artery) and hence this constitutes a non-peptide antagonist of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) (24). The NK_1 receptor affinity within this series of quinuclidines is reportedly determined by an unsubstituted benzhydryl group, the bridgehead nitrogen and the benzylamino side-chain. These features have been incorporated into a 3-point NK_1 receptor binding model (25). In in vivo assays, 12 is a potent inhibitor of substance P mediated neurogenic inflammation in rats and an inhibitor of substance P mediated airway plasma exudation in guinea-pigs (26,27). The biological data generated to date with CP-96345 are consistent with the hypothesis that substance P is involved in the transmission of noxious stimuli within the CNS and a substance P antagonist may be useful as a novel analgesic/antiinflammatory drug. However, the use of this compound as a selective NK_1 ligand is limited because it interacts with calcium channels (28). This affinity is not present in the more recently reported derivative

(CP-99994, **13**) which has a lower molecular weight and appears to be a more selective NK_1 antagonist (29). Other NK_1 selective non-peptides identified using a broad screening approach have been reviewed previously (30).

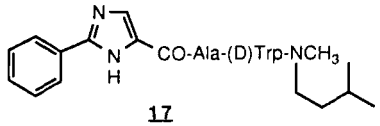
In addition to the successful identification of non-peptide NK_1 antagonists based on the above broad screening approaches, there have also been reports of "design" strategies in which a peptide provides the initial chemical lead. One such antagonist is CGP 47899 (**14**) (31) and the general strategy utilized involves: i) identify which groups (within the peptide) are important for receptor binding; ii) investigate the 3-D arrangement of these groups; and iii) attach the key groups in an appropriate orientation to a rigid, non-peptide template. In this example the previously reported substance P peptide SAR data led to the hypothesis that the Phe^7-Phe^8 dipeptide subunit of substance P is particularly important for receptor binding and Z-Phe-OBzl was identified as a medium affinity small molecule lead with three lipophilic side-chains. Similar affinity was also observed for piperazine derivatives with 2 or 3 similar lipophilic groups appended. Subsequent SAR studies involving manipulation of the piperazine template led to CGP 47899 in which one of the N-atoms is exocyclic (31). Both this NK_1 strategy and that which independently led to the CCK-B antagonist CI-988 identify a small molecule fragment of the endogenous peptide with weak binding affinity as a key chemical lead.



15



16



17

The design of FK 888 (**15**) (see Fig. 2) started from a previously reported octapeptide substance P antagonist derivative, **15a**. Simplification of this structure was achieved by testing six continuous tripeptide fragments (i.e., D-Pro-Gln-Gln, Gln-Gln-D-Trp etc.). This led to **15b**. Replacing the N-terminal residue of this tripeptide and modifying the N- and C-terminal "protecting groups" produced **15c**, a compound with significantly increased affinity. The next step was to decrease the molecular weight by removing the least important group (Gln-). Subsequent modification of the peptide bonds led to the dipeptide derivative, FK 888. This compound has high affinity and antagonist activity at the NK_1 receptor. Furthermore, FK 888 inhibits substance P induced airway edema in guinea-pigs after oral and iv administration (32, 33). The biological activity of this and other non-peptide NK_1 antagonists (and the neurotensin and bombesin antagonists in table 1) has been shown to be species selective. The rodent data are not necessarily predictive for primates.

Figure 2. Summary of Discovery of FK 888, 15, (32, 33)

	NK_1 receptor binding affinity (guinea-pig lung, IC_{50})*
D-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-Phe-NH_2 (15a)	600 nM
↓	
D-Trp-D-Trp-Phe-NH_2 (15b)	10,000 nM
↓	
Boc-Gln-D-Trp(CHO)-Phe-OBzl (15c)	90 nM
↓	
FK 888 (15)	0.69 nM

*These binding data are obtained in the same laboratory.

Neurokinin - 2 - The first non-peptide NK_2 selective antagonist to be reported is the 4-phenylpiperidine derivative SR 48968 (16). It displaces a radiolabelled sample of the endogenous peptide neurokinin A (NK-A)(His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) from rat duodenum and hamster urinary bladder membranes and behaves as an antagonist in isolated smooth muscle NK_2 preparations. In vivo SR 48968 (16) antagonises NK-A induced bronchoconstriction in guinea-pigs (34). The chemical strategy leading to the identification of this compound has not been disclosed to date.

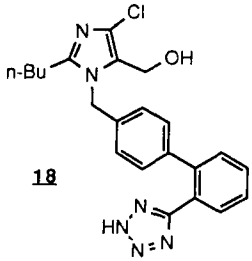
GR 112000 (17) was designed from a heptapeptide antagonist (see fig. 3). Its conversion into a fully non-peptide NK_2 antagonist has not been reported yet.

Figure 3. Summary of development of the NK_2 receptor antagonist, GR 112000 (17) (35).

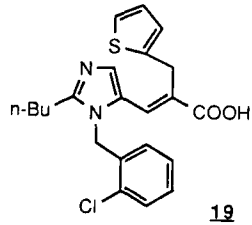
	mol. wt.	NK_2 receptor antagonism pK_B (rat colon)*
PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2	904	9.56
↓		
PhCO-Ala-Ala-D-Trp-Phe-NMe_2	625	8.56
↓		
PhCO-Ala-Ala-D-Trp-NMe_2	478	6.45
↓		
PhCO-Ala-Ala-D-Trp-NMe($CH_2CH_2CHMe_2$)	534	8.00
↓		
GR 112000 (17)	528	7.64

*These efficacy data are obtained in the same laboratory.

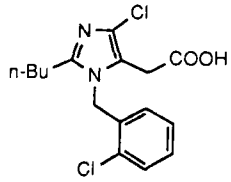
Angiotensin II - Both the AT_1 and the AT_2 receptor subtypes are present in the brain, although their therapeutic role in the CNS is unclear. Previous reviews of non-peptide selective AT ligands have comprehensively surveyed the cardiovascular effects (36-38). The structure of losartan (DuP 753) continues to provide the inspiration for many medicinal chemists to synthesize even more potent, orally active AT_1 antagonists. For example ICI D6888 retains the biphenyltetrazole moiety of DuP 753 (18) but replaces the imidazole with a 4-alkoxypyridine which is proposed to mimic the hydrogen-bonding properties (39). The



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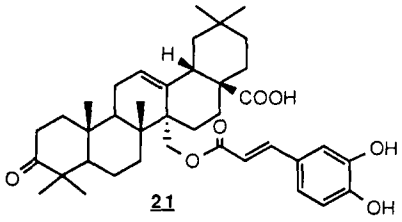


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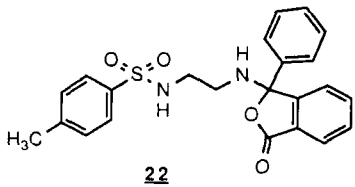


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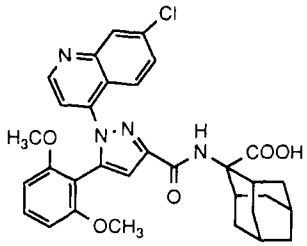
strategy that led to compounds of the type 19 has recently been disclosed (40) : the initial non-peptide AT lead (20) was overlayed onto a postulated pharmacophore model of the endogenous octapeptide. From this it was proposed that the imidazole ring of 20 acts as a template to orient mimetics of the Tyr^4 and Ile^5 side-chains and the C-terminal COOH of AT (the substituted phenyl, n-Bu and COOH groups on 20 respectively). Structural modifications made to the non-peptide to improve this overlay led to 19 which exhibits a significant increase in AT_1 antagonist activity when compared with 20 (40). This success was achieved using a molecular modelling overlay strategy together with judicious use of peptide SAR data.



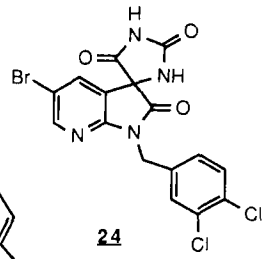
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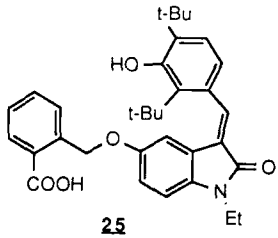
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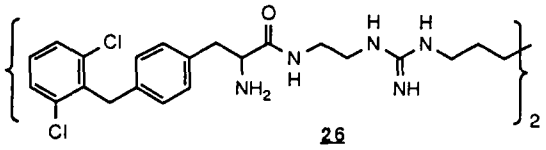


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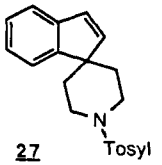
Endothelin, Neurotensin, Bombesin, Neuropeptide Y, Oxytocin, Opioid - Recently reported non-peptide ligands for these receptors are shown in table 1, together with brief biological data. Apart from the opioids (50), the full pharmacological characterisation of these compounds has not yet been reported. Previously described non-peptides have been surveyed (30,45).

Table 1. Examples of Non-Peptide Ligands for Neuropeptide Receptors and the Chemical Strategy used for their Discovery.

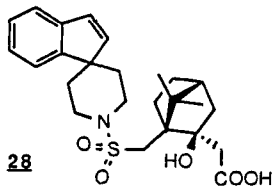
Neuropeptide Receptor	Non-Peptide Ligand	Chemical Strategy used for the Discovery	Binding Affinity	Reference
CCK-A	Devazepide 2	natural product broad screen then lead optimisation	0.8 nM	6
	PD-140548, 9	modification of literature lead (CI-988 series)	2.8 nM	15
	SC-50998, 10	pyrrolidinone template	16 nM	16
CCK-B	L-365260, 3	natural product broad screen then lead optimisation	2.0 nM	6
	quinazolinone, 4	natural product broad screen then lead optimisation	9.3 nM	9
	LY-262,691, 5	broad screening and lead optimisation	31 nM	10
	CI-988, 6	designed from endogenous peptide	1.7 nM	11
NK_1	CP 96345, 12	broad screening then lead optimisation	0.6 nM	24,25
	CP 99994, 13	modification of literature lead (CP 96345)	0.17 nM	29
	CGP 47899, 14	designed using peptide SAR and template approach	30 nM	31
	FK 888, 15	designed from octapeptide antagonist	0.69 nM	33
NK_2	SR 48968, 16	not reported	0.5 nM	34
	GR 112000, 17	designed from heptapeptide antagonist (pK_B = 7.64)	-	35
AT_1	DuP 753, 18	modification of literature lead	12 nM	38
	19	overlay of literature lead and endogenous peptide	440 nM	40
Endothelin ET_A	myriceron caffeoyl ester, 21	natural product screen (antagonist in vitro pA_2 = 6.65)	78 nM	41
Neurotensin	UK 73093, 22	broad screening (rat selective)	5.5 μM	42
	SR 48692, 23	not reported	20 nM	43
Bombesin	CP 75998, 24	broad screening (rat selective, antagonist in vitro)	1.5-3 μM	44
	CP 70030, 25	broad screening (rat selective, antagonist in vitro)	1.5-3 μM	44
Neuropeptide Y	26	modification of literature lead (antagonist in vitro)	35 μM	46
Oxytocin	27	broad screening	4 μM	47
	L-366509, 28	optimisation of 27 (antagonist in vivo)	480 nM	47
Opioid	Morphine, 1	natural product (mu, δ, K agonist in vivo)	-	-
	CI-977, 29	modification of literature lead (spiradoline) (K-agonist in vivo)	0.11 nM	48,49



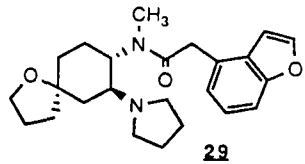
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29

Summary and Future Directions - Currently the two most successful strategies for the discovery of non-peptide antagonists for neuropeptide receptors are i) broad screening of natural products or chemical files in an automated receptor binding assay usually followed by chemical lead optimization, and ii) "design" starting from the peptide structure of a known antagonist or the endogenous agonist. Key steps in this latter strategy include one or more of the following :

a) utilize peptide structure-affinity (or activity) data to identify key amino acid side-chains,
b) append key amino acid side-chains onto a non-peptide template (or scaffold),
c) identify a mono, di- or tri-peptide with medium binding affinity (micromolar),
d) enhance the affinity of a weakly active lead by incorporating conformational restraint.

To date the only class of non-peptides for which clear receptor agonist data have been reported are the opioids and these have been comprehensively surveyed elsewhere (50).

The strategies described in this chapter should facilitate the discovery of non-peptide ligands for other neuropeptide receptors and in principle they may also be applied to additional receptor targets for which there are peptide ligands, e.g. growth hormone receptors or ion channels. Molecular modelling may play an increasingly important role in the design strategies as knowledge of the molecular structure of receptor binding sites becomes more detailed.

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SECTION II. CARDIOVASCULAR AND PULMONARY AGENTS

Editor: David W. Robertson, Ligand Pharmaceuticals
San Diego, CA 92121

Chapter 8. Hypertension Therapy: What Next?

R. Allan Buchholz, Bruce A. Lefker, M.A. Ravi Kiron
Pfizer Inc., Central Research Division, Groton, CT 06340

Introduction - Advances in antihypertensive therapy in the past three decades have significantly reduced cerebrovascular accidents and improved overall cardiovascular morbidity and mortality (1,2). This finding, coupled with the availability of several types of antihypertensive agents (3) and current economic trends, may raise skepticism over the need for new antihypertensive drugs. Similar sentiments were expressed prior to the discovery of angiotensin converting enzyme (ACE) inhibitors and calcium antagonists (1). However, treated hypertensive patients still have increased risk of morbid and fatal events associated with coronary artery disease (CAD), the major cause of death in Western societies (2).

Essential hypertension can no longer be considered simply a state of elevated blood pressure. It is a syndrome of multiple abnormalities often characterized by cardiac and vascular restructuring (4), lipid abnormalities (5) and defects in carbohydrate metabolism associated with insulin resistance/hyperinsulinemia (6). New antihypertensive therapy will need to control hypertension more effectively, with fewer side effects, once-a-day dosing and neutral/positive impact on known cardiovascular risk factors (1,2). Requisite morbidity-mortality trials should be initiated early to establish the advantages of new agents.

The renin-angiotensin system (RAS) continues to be a major focus for antihypertensive therapy. The molecular biology of this system and the significance of the tissue RAS in cardiovascular disease has been summarized (7,8). The success of ACE inhibitors in controlling blood pressure and improving mortality in heart failure (9,10) is noteworthy. The identification of two active sites on ACE cloned from human endothelium suggests possible differential affinities for ACE substrates and the potential for differential inhibition of the angiotensin and bradykinin pathways, i.e., a selective ACE inhibitor (11). Substantial effort continues to be directed toward the development of RAS antagonists (renin inhibitors and angiotensin II (AII) receptor antagonists) that may have better efficacy and/or side-effect profiles than ACE inhibitors. Development of selective, nonpeptide AII receptor antagonists continues to be an active area of antihypertensive drug discovery. New therapeutic targets in the RAS cascade are emerging, including potential applications of gene therapy (12). The link between insulin resistance, hyperinsulinemia and hypertension is currently a very active area in hypertension research. This area was highlighted by the recent disclosure of the antihypertensive actions of two distinct classes of agents that enhance insulin sensitivity. Antihypertensive agents targeted at second messenger systems may allow regulation of multiple endpoints in the hypertensive process. Protein kinase C is an important regulator of vascular tone, vascular and cardiac growth, fluid balance and blood pressure. The imidazoline receptor, distinct from the α-2 receptor, is an emerging pharmacologic target for the central nervous system regulation of hypertension and its complications (13). The status of these current and potential drug discovery targets for hypertension therapy are summarized below.

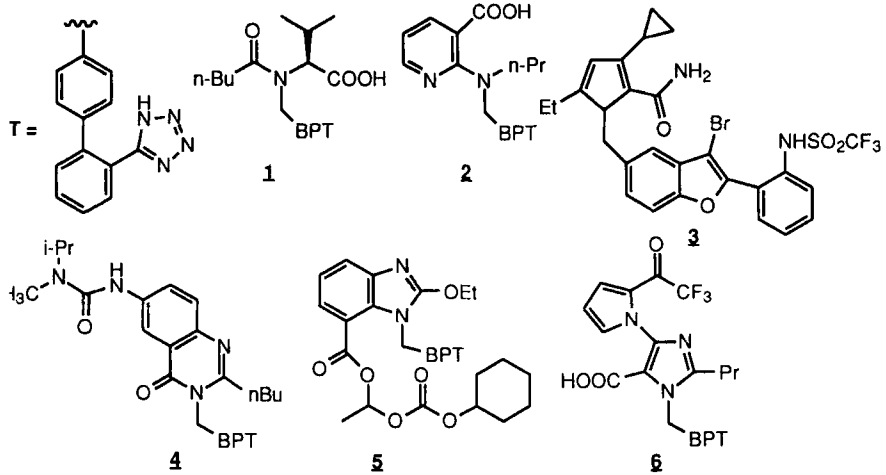
RENIN-ANGIOTENSIN MODULATORS

AII AT_1-receptors - Recent reviews describe an extensive array of new structural types of AII, AT_1-selective receptor antagonists. (14-16). With the exception of SKF-108566, recently introduced structures are predominantly based on modifications to one or more fragments of losartan (DuP-753). A large number of heterocyclic replacements for the imidazole of losartan have appeared. Recent disclosures include, dihydropyrimidines (17), oxadiazinones (18), and benzothiadiazine dioxides (19). Structures have also appeared in which the imidazole headpiece is replaced by a tri- or disubstituted amine (20). Valsartan (**1**) and A-81,988 (**2**) are examples of potent, orally active antagonists at different stages of development (21,22). An area of recent interest has been the search for orally active compounds which lack the acidic tetrazole (or carboxylic acid) of losartan. A variety of alternative groups such as trifuoromethylsulfonamides (GR138950, **3**), oxadiazoles and sulfonyl ureas have been used to introduce an acidic moiety into the biaryl unit (23, 24, 25, 26).

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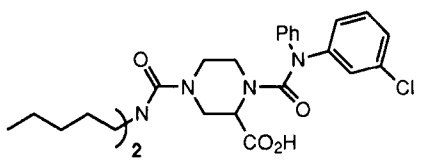
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However, good oral efficacy has only been reported with **3**. Continued interest in replacement of one of the phenyl groups of losartan's biphenyl tetrazole (BPT) with various heterocycles led to the orally active pyridine derivative SC52,458 (27). Other promising compounds which have good oral activity are BIBR277, L-159,093 (**4**), TCV-116 (**5**) and CI-996 (**6**) (28, 29, 30, 31).



Extensive reviews on the pharmacology of nonpeptide AII receptor antagonists have appeared (32,33). The AT_1-receptor cDNA's from bovine, rodent and human tissues were cloned (33). Two structurally distinct isoforms of the AT_1-receptor (termed AT_{1a} and AT_{1b}), were identified in the rat (34,35) and mouse genome (36); only one form of the AT_1-receptor was found in human tissue (37). The functional attributes of the cloned AT_1-receptor subtypes have not been fully determined. The antihypertensive effects of losartan may not be due solely to AII receptor antagonism (38). Inhibition of nitric oxide (NO) synthesis blunted the hypotensive effect of losartan, suggesting NO may contribute to the antihypertensive action of this agent (39). Studies of AT_1-receptor antagonists in various models of heart failure demonstrated beneficial hemodynamic effects (40-42) and prevention of cardiac hypertrophy (43). The hypotensive response to AT_1-receptor antagonism was enhanced by combined delivery with an AT_2-receptor antagonist (44, 45). This may be a consequence of AT_2-antagonist displacement of plasma protein bound AT_1- antagonist, resulting in enhanced free drug concentration of the latter (44). Emerging clinical findings with losartan indicate a dose range for antihypertensive therapy of 40-80 mg/day (46-48). Losartan has demonstrated a distinct clinical advantage over ACE inhibitors in hypertensive patients by minimizing the incidence of cough (not different from placebo), a significant side-effect of chronic ACE-inhibitor therapy (48). Evaluation of losartan in heart failure patients showed positive hemodynamic and neurohormonal effects (49).

AII AT_2-receptors - Identification of multiple receptor subtypes for AII and the emergence of inhibitors specific for either the AT_1 or AT_2-receptor subtype have been described (9,33). AT_2-binding sites have been localized in a number of tissues, as well as in cells cultured from different species (50). The AT_2 - receptor has not been cloned. A number of new non-peptidic antagonists of the AT_2 binding site have been reported. Compounds such as BIBS 39 and BIBS 222 inhibit both the AT_1- and AT_2 -receptor subtypes (51); others, such as L-159,874 (**7**), are reportedly specific for the AT_2-receptor subtype (52). Specific ligands, such as the radiolabelled CGP 42112A, have revealed the presence of multiple subtypes of the AT_2-binding site (53, 54). Acute antagonism of the AT_2-receptor alone does not lead to the antihypertensive effects seen with AT_1-receptor antagonists. However, reflex increases in plasma AII that occur with AT_1- antagonism may cause over stimulation of the AT_2 - receptors. During prolonged blockade of the vasoactive AT_1- receptor, some of the hemodynamic and/or mitogenic effects of AII may be mediated through



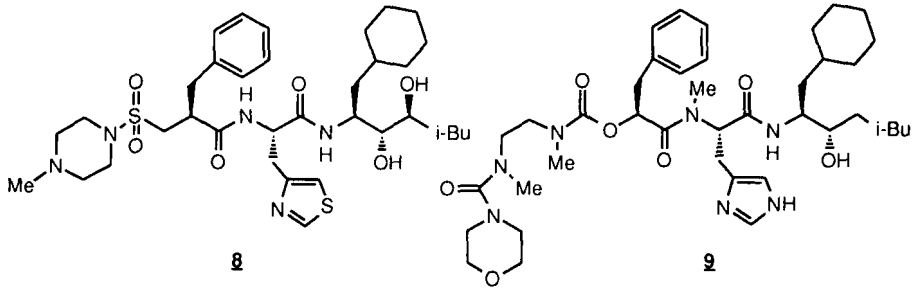
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the AT_2-receptor (45). Combined antagonism of both receptor subtypes may have therapeutic advantages over selective AT_1-receptor antagonism.

The function of the AT_2-receptor is presently unclear. Studies describing the biochemical responses to AII binding at AT_2-receptors on PC12W cells (55), and NG108-15 cells (56) have appeared. A role for the AT_2-receptor in the regulation of vascular smooth muscle cell proliferation (57), cGMP production (58) and cerebral blood flow (59) have been reported. Antagonism of the AT_2- but not the AT_1-receptor suppresses collagen synthesis by human cardiac fibroblasts (60). The depressor phase of the biphasic response to angiotensin III is mediated by AT_2 - receptors and is dependent on nitric oxide release (61). A significant increase in AT_2 - receptor density occurs around the healing wound (62). Studies describing the efficacy of both AT_1- and AT_2-antagonists in regression of myocardial hypertrophy have appeared (56,63). AT_2 -antagonists have been claimed as potential agents for the treatment of a number of CNS disorders (64).

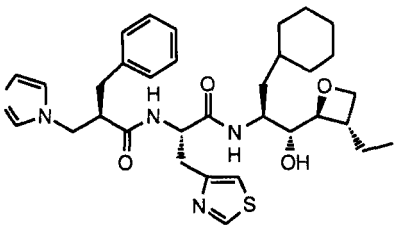
Other AII receptor subtypes - Additional functionally coupled AII-receptor subtypes, with high affinity for AII, have been identified that are not responsive to antagonism with either losartan or PD 123319, a selective AT_2 - receptor antagonist (65, 66). The soluble angiotensin-binding protein has been cloned; it has no homology with the AT1 receptor or other known proteins (67). It is present in the cytosol and the nuclear plasma(68). A new binding site specific for the C-terminal 3-8 hexapeptide fragment of angiotensin II (referred to as AIV) has been discovered (69, 70). This binding site displays low affinity for AII, AIII and Sar^1,Ile^8-AII. It is not affected by the nonpeptidic AT_1- or AT_2-receptor antagonists and is a potent stimulator of renal cortical blood flow (69).

Renin - Various aspects of the renin-angiotensin system have recently been reviewed (7, 8, 16, 71). Unfortunately, the development of renin inhibitors as successful therapeutic agents has been hindered by poor pharmacokinetics and low oral bioavailabilities. Two compounds with improved oral bioavailabilities are now undergoing clinical evaluation. A 50 mg oral dose of A-72517 (**8**) to furosemide-treated normotensives led to a significant reduction in blood pressure (72). High bioavailability after i.d. dosing with this compound was observed in animal models (73). Good oral bioavailability and blood pressure lowering at 50 mg bid in humans has also been reported with FK-906 (**9**) (74, 75). New clinical data with previously disclosed renin inhibitors has also been reported during the past year (76, 77). Various formulations and routes of administration have been utilized to improve exposure to SC-46944 (78). Comparisons of renin inhibitors with ACE inhibitors in heart failure models demonstrated similar beneficial hemodynamic changes with both classes of agents (79, 80).

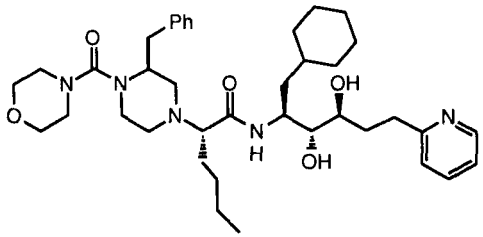


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An oxetane-containing, transition-state mimic with increased affinity for renin has facilitated the identification of potent inhibitors with reduced molecular weights (**10**) (81). Substituted cyclopropanes have been explored as conformationally restricted phenylalanine analogs (82). Compounds which introduce solubilizing groups (83, 84), stabilize or remove labile amide bonds as in **11** have been reported (85, 86). A hydrophilic, pentol renin inhibitor with improved bioavailability may be transported by the same transporter as the β-lactam antibiotics (87). Difluoroketone containing renin inhibitors have shown good oral activity in cynomolgus monkeys (88). Modest oral efficacy was observed in a series of inhibitors with α-heteroatoms at P_2 (89). Compounds with certain P4 groups were found to be slow, but reversible inhibitors of human renin (90). Also, X-ray structures of mouse and human renin complexed with peptidic inhibitors have been reported (91).

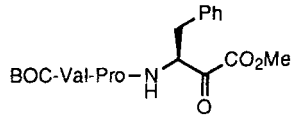


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Chymase - ACE, a dipeptidyl carboxylase, is a key enzyme in forming angiotensin II (AII) from angiotensin I (AI) (92). In many tissues, the conversion of AI to AII still occurs in the presence of ACE inhibitors (93-96). One enzyme apparently responsible for the non-ACE production of AII is inhibited by chymostatin, an inhibitor of chymotrypsin-like enzymes (94, 95, 97, 98). DNA analysis indicates that this enzyme belongs to the class of enzymes called chymases (99). This highly specific chymase converts AI to AII without further degradation, and with greater efficiency and selectivity than ACE (97, 100). Angiotensin I converting chymase activity has a wide tissue distribution in nonhuman primates (100). Its functional role in the *in vivo* production of AII is unknown, however, the biochemical characteristics of this enzyme are compatible with its participation in an alternate pathway for conversion of AI to AII. Several pharmacologic tools are emerging that may validate the role of chymase as an important enzyme in the renin-angiotensin cascade and demonstrate its potential as a therapeutic target. A putatively selective substrate, (Pro^{11}, dAla^{12})-AI, for this enzyme has been identified (101). Administration of the substrate to ACE-blocked baboons caused an AII-mediated increase in blood pressure (101). CP-162,213 (12) is a potent inhibitor of chymase (IC50 = 30nM)(102). It is selective for chymase with an IC50 > 1.0 μM for trypsin and cathepsin G, and greater than 100 μM for elastase. It does not inhibit renin or ACE activity, nor does it affect AII binding.



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GENES AS THERAPEUTIC TARGETS

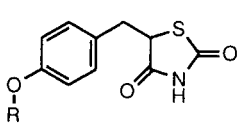
Gene mapping/transgenic models - Hypertension is a complex disorder arising from the interplay of environmental and genetic factors. A clear understanding of the genetic foundations of hypertension may offer a new approach to the prevention or reversal of this disease process. Use of gene mapping/linkage analysis (association between the disease and a marker genotype, i.e. polymorphism) and transgenic animal models may reveal candidate genes as therapeutic targets. To date, genetic linkages have been demonstrated between the angiotensinogen (Ao,103) and ACE (104) genes and human hypertension. No correlation has been established between polymorphisms of the human renin gene and hypertension (105). A deletion polymorphism in the ACE gene has been identified as a risk factor for myocardial infarction in humans, but was unrelated to hypertension (106). The development of transgenic technology has allowed assessment of the effects of precise genetic modifications *in vivo*; studies are presently directed at evaluating a severely hypertensive transgenic rat model harboring the mouse renin-2 gene (107).

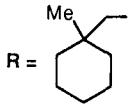
Gene therapy - The goal of gene therapy is to: 1) correct a genetic defect through replacement of defective gene sequences in target tissues; 2) introduce exogenous or endogenous genes to correct a genetic defect; or 3) enhance or suppress expression of an extant protein within a diseased individual. The identification of candidate genes involved in hypertension provides potential targets for gene therapy. The ability to introduce and express recombinant genes *in vivo* through indirect and direct gene transfer in vascular (108) and cardiac (109) tissue has established the feasibility of gene therapy for cardiovascular disease. Antisense technology (110), has the potential of using oligonucleotides to inhibit or control gene expression with greater specificity and efficiency than is offered by traditional, protein-based therapeutics (111). Permanent inhibition of Ao gene expression

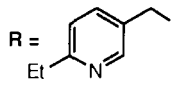
in cultured cells using the antisense approach has been reported (112). Future strategies may also involve introduction of genes encoding mutant proteins (such as mutant Ao) that would be constitutively produced and act as endogenous antagonists of their normal protein counterpart (113).

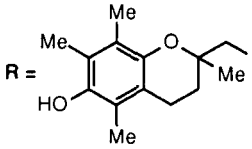
INSULIN RESISTANCE AND HYPERTENSION

The relationship between insulin-resistance and hypertension has become one of the most active areas of hypertension research (114-116). A constellation of related variables ('Syndrome X') associated with hypertension, diabetes, obesity, atherosclerosis and CAD has been described (117). Insulin-resistance is the basic common feature of Syndrome X from which the other changes (hyperinsulinemia, hypertension, glucose intolerance, increased very low density lipoprotein triglycerides and decreased high density lipoprotein cholesterol) are likely to emanate. Insulin-resistance has been found in lean, nondiabetic essential hypertensives and is directly correlated with the severity of hypertension (6). Insulin-resistance persists during antihypertensive therapy (118), suggesting that it is not secondary to hypertension. Insulin-resistance has also been demonstrated in salt-sensitive, otherwise healthy, normotensive individuals and may be indicative of future cardiovascular disease in this population (119). Multiple actions of insulin have been proposed to explain the association between insulin resistance and hypertension, including increased sympathetic nervous system activity, increased sodium reabsorption, altered cation transport and proliferation of vascular smooth muscle cells (114). The mechanism(s) responsible for insulin-resistance is unknown. Current knowledge favors a post-receptor defect (118), although there also appears to be a hemodynamic basis for insulin-resistance (120-122). Animal models of insulin-resistance and hypertension have been described (123-125). Current antihypertensive therapy (ACE inhibitors, α-blockers) results in only modest improvements in insulin sensitivity and plasma insulin levels (118, 126).









13 14 15

Several agents from two classes of drugs (biguanides and thiazolidinediones) with insulin enhancing action (127) have demonstrated antihypertensive properties. Non-obese, non-diabetic hypertensive men given metformin, a biguanide, exhibited significant reductions in blood pressure, total and LDL-cholesterol, triglycerides and plasma insulin levels that returned to pretreatment values upon withdrawal of the treatment (128). Similar results were observed with metformin in normotensive noninsulin-dependent diabetic patients (129) and in hypertensive animals (130). The thiazolidinediones, ciglitazone (ADD-3878,**13**), pioglitazone (ADD-4833, **14**) and CS-045 (**15**), lowered blood pressure and improved insulin profiles in several rodent models of hypertension (131-133). Both **13** and **14** also inhibited the mitogenic effects of growth factors on cultured vascular smooth muscle cells (131, 132). The relationship between the antihypertensive and insulin sensitizing actions of metformin and the thiazolidinediones is unknown. However, they may represent intriguing new classes of antihypertensive compounds.

SECOND MESSENGERS AS THERAPEUTIC TARGETS

Protein kinase C - Protein kinase C (PKC) has emerged as a major signal transduction system activated by a variety of external stimuli impinging on the cell membrane (134, 135). Originally isolated as a single cytosolic, proteolytically activated enzyme from brain, PKC now represents a family of 8-10 isozymes with distinct structural and enzymatic properties and differential tissue distribution (134-136). Agonist-induced, G-protein coupled hydrolysis of phosphatidylinositol promotes mobilization of intracellular calcium, generation of diacylglycerol (DAG) and activation of PKC (137). PKC activation results in phosphorylation of serine/threonine residues on target proteins and is intimately involved in cell regulatory functions. Functional heterogeneity of PKC isozymes is suggested by differences in tissue distribution, substrate specificity, calcium requirements and phospholipid dependency (134-136).

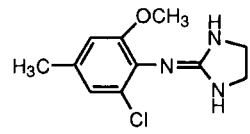
The pivotal role of PKC in signal transduction makes it a logical target for drug intervention (138, 139). Discovery of specific PKC inhibitors as antineoplastic agents has received considerable

attention because of PKC regulation of cell growth (139). This same attribute may contribute to the usefulness of PKC inhibitors in modulating vascular and cardiac remodeling in hypertension and heart failure. Potent and specific inhibitors of PKC have been synthesized or derived from microbial sources (139,140 for reviews). Staurosporine, one of the most potent inhibitors available, and H7, an isoquinolinesulfonamide, are two of the most commonly used PKC inhibitors, but lack selectivity because they compete for the ATP binding site on the catalytic domain of PKC, a region highly conserved across protein kinases (140). In contrast, calphostin C represents a new class of inhibitors interacting with the regulatory domain of PKC, which is distinct from that of other protein kinases (140). Synthesis of isozyme-specific PKC inhibitors represents a significant and unmet challenge.

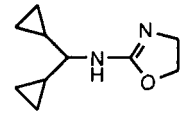
Activation of the PKC signalling pathway by a variety of agonists, including angiotensin II, has been implicated in the stimulation of vascular and cardiac cell growth (proliferation and hypertrophy, 141-144), maintenance of vascular smooth muscle contraction (145-147) and regulation of renal function (148), all of which may contribute to the hypertensive process. PKC, either directly or upon activation by AII, inhibits atrial natriuretic peptide (ANP)-stimulated guanylate cyclase activity and cyclic guanosine monophosphate (cGMP) accumulation in vascular smooth muscle (149) and renal glomerular (150,151) cells. This suggests that activated PKC may directly oppose the vasorelaxant and natriuretic actions of cGMP *in vivo*. Inhibition of PKC with potent, nonselective inhibitors such as staurosporine relaxes vascular smooth muscle (146, 147, 152), lowers blood pressure in normotensive rats (153) and dogs (154), and hypertensive rats (152, 155). Enhanced contractile responsiveness to PKC activators (156) and increased basal and stimulated PKC activity (157) have been reported in spontaneously hypertensive rats. PKC has also been reported to contribute to endothelin-1-stimulated increases in vascular myofilament calcium sensitivity (158) and the occurrence of chronic vasospasm in dogs after subarachnoid hemorrhage (159). The specific PKC isozyme(s) involved in these responses is unknown.

CENTRALLY ACTING ANTIHYPERTENSIVES

Until recently, the sympatholytic action of clonidine and other centrally acting antihypertensives was believed to arise from stimulation of brainstem α-2 adrenergic receptors. The discovery of a nonadrenergic binding site and its correlation with the antihypertensive activity of agents such as clonidine has led to the development of a second generation of centrally acting drugs that bind to the I$_1$-imidazoline receptor (160, 161). The primary locus of action of I$_1$-receptor agonists is in the rostral ventrolateral medulla of the brainstem, the putative site of origin of sympathetic nervous system activity (162). The imidazoline receptor protein has been isolated but has not been cloned (163). Two recently introduced imidazolines, moxonidine (**16**) and rilmenidine (**17**), retain the antihypertensive efficacy of clonidine but cause significantly less incidence of dry mouth and sedation (164, 165). The sedation and dry mouth caused by clonidine are ascribed to activation of α-2 receptors. The relatively low affinity of **16** for the α-2 receptor (40-200 fold ≤ I$_1$-receptor) may account for its reduced incidence of side-effects (161). In addition to their antihypertensive actions, once-a-day monotherapy with **16** (0.2-0.4 mg) and **17** (1-3 mg) lowered plasma catecholamines and renin activity, reversed cardiac hypertrophy, caused less bradycardia than clonidine, demonstrated no rebound hypertension and behaved neutrally towards plasma glycolipid metabolism (164, 165). These characteristics suggest that potent, selective imidazoline receptor agonists will have significant utility in the treatment of congestive heart failure, as well as hypertension.



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Conclusions - The explosion of information on the RAS continues to provide potential new targets (e.g., chymase) for drug discovery, but demonstration of significant advantages over existing therapy is a difficult challenge. However, combined sequential inhibition of enzymes or receptors in the RAS cascade may demonstrate improved efficacy and safety over that of individual inhibitors (166). Agents that are antihypertensive and significantly improve risk factors associated with insulin-resistance would have breakthrough potential in the treatment of hypertension. Gene transfer strategies hold distant promise and may be most useful in combination with typical pharmacological

antihypertensive therapy (167). The complex nature of second messenger systems, such as the PKC pathway, render selective drug action difficult. Whether it is too complex for successful therapeutic intervention remains to be seen (139). The discovery of the imidazoline receptor sets a new hallmark for targeting the central nervous system in the treatment of hypertension and heart failure.

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CHAPTER 9. GLYCOPROTEIN IIbIIIa ANTAGONISTS

Brent K. Blackburn and Thomas R. Gadek
Department of Bioorganic Chemistry
Genentech, Inc
South San Francisco, CA, 94080

Introduction - The platelet glycoprotein IIbIIIa (GPIIbIIIa) is a member of the integrin family of adhesion receptors and is involved in the adhesive events leading to platelet aggregation and thrombus formation (1,2). Naturally occurring ligands for GPIIbIIIa include fibrinogen (3), fibronectin (4), vitronectin (5), von Willebrand factor (6) and thrombospondin (7). While each of these ligands are capable of supporting platelet adhesion *in vitro*, the binding of fibrinogen to GPIIbIIIa is the ultimate event leading to platelet aggregation *in vivo* (8). In this review, we will summarize work leading to the discovery of antagonists of GPIIbIIIa/fibrinogen interactions as antiaggretory and antithrombotic agents for treatment of human vascular diseases (9,10).

Glycoprotein IIbIIIa is a noncovalent heterodimeric protein complex whose tertiary structure is dependent on the association of divalent cations (11-13). Each subunit has a single transmembrane domain and a short cytoplasmic tail (14). For some time, GPIIbIIIa and other integrins were thought to have a purely adhesive function, and as such were considered incapable of signal transduction. However, recent work has shown integrins in general, and GPIIbIIIa in particular, are involved in both inside-out and outside-in signaling (15,16). In addition to these signaling and adhesive functions, GPIIbIIIa is involved in the trafficking of proteins from serum into the platelet *alpha*-granules (17-19). A number of reports have implicated GPIIbIIIa in the transport of calcium ions into the platelet upon activation. However, this may be an indirect effect since GPIIbIIIa does not appear to be a calcium channel (20).

Upon activation of platelets by any number of stimuli, morphologic and biochemical changes occur. Platelets initially change shape from elliptical to spherical, increase their surface area, develop projections which are rich in GPIIbIIIa as well as fibrinogen and become capable of aggregation (18,21). Concurrently, in the cytoplasm, various proteins are phosphorylated by tyrosine kinase(s) (22) and protein kinase C. In addition, intracellular calcium levels rise, cyclic AMP levels decrease (23), the cytoplasmic domain of GPIIbIIIa associates with cytoskeletal elements and the extracellular domain of GPIIbIIIa acquires an enhanced affinity for fibrinogen (24-26). The precise time course of these events is still unclear, but the overall process is fast and is generally measured in seconds. The affinity of GPIIbIIIa for fibrinogen can also be modulated by the lipid composition of the platelet membrane (27) as well as by partial proteolysis of the extracellular domain (28).

In the absence of stimulation, platelets are still capable of adhering to fibrinogen or other adhesive proteins that are immobilized on a solid surface (15). This adhesion can be mediated by either GPIIbIIIa/fibrinogen association, or the association of glycoprotein Ib with von Willebrand factor (29). Both cases are relevant to the development of thrombotic vascular occlusions *in vivo*.

Deficiencies in the synthesis (30-33), transport, or surface expression (34) of GPIIbIIIa as well as the formation of antibodies against GPIIbIIIa (35) result in lifelong bleeding disorders classified as Glanzmann's thrombasthenia or chronic immune thrombocytopenia purpura. Glanzmann's thrombasthenia manifests itself in bleeding from gums and mucotaneous tissue and is not typically life threatening even when no functional GPIIbIIIa is expressed (31). No other untoward clinical effects are exhibited in these patients.

PROTEINS

Antibodies- Numerous antibodies have been prepared against the GPIIbIIIa complex as well as against the individual subunits. They have been used to map binding epitopes (36,37), assess the activation state of the GPIIbIIIa (38), activate platelets toward aggregation (39), and block fibrinogen binding (40). A series of non-neutralizing antibodies prepared against the high affinity state of GPIIbIIIa have been observed to stimulate resting platelets to aggregate in the absence of exogenous stimulants (41), a result consistent with the antibody arresting the high affinity state of GPIIbIIIa (41). Anti-idiotypic antibodies against the anti-GPIIbIIIa antibody PAC1 appear to mimic the function of GPIIbIIIa by binding fibrinogen (42).

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Of the monoclonal antibodies capable of blocking GPIIbIIIa/fibrinogen interactions, 7E3 is the most widely studied (43). This antibody prevents platelet aggregation and thrombosis in a canine model of coronary angioplasty (44). Additional studies have demonstrated enhanced lysis rates and lower incidence of reocclusion in animal models of arterial thrombolysis (45-47).

Disintegrins- A number of low molecular weight proteins capable of inhibiting GPIIbIIIa/fibrinogen association have been isolated and characterized from snakes and leeches, and have been given the name disintegrins (48). Members of the snake venom family of proteins include echistatin (49), kistrin (50), barbourin (51), and trigramin (52) and are related to the leech proteins decorsin (53), and ornatin (54). These proteins are potent inhibitors of platelet aggregation *in vitro* and have similar sequence and disulfide patterns (55). The most striking sequence similarity is that with the exception of barbourin, they all have the tripeptide sequence Arg-Gly-Asp (RGD), crucial to many integrin/ligand interactions (1). Barbourin has the conservative change to Lys-Gly-Asp (KGD) at a homologous region in the protein. This sequence confers selectivity for GPIIbIIIa over related integrins such as the vitronectin receptor (56).

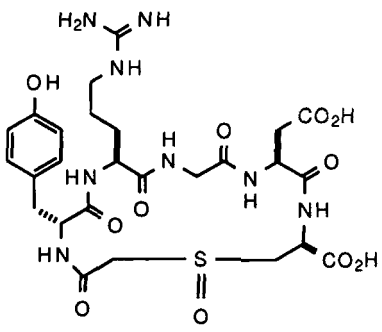
Recently, the solution structures of kistrin (57) and echistatin (58-61) have been solved by two-dimensional NMR studies. Both proteins display the RGD sequence in large loops extending away from the bulk of the protein. Neither structure exhibited a well-defined RGD conformation. A structure has been proposed for mambin, another snake venom protein with sequence and structural homology to erabutoxin b (62).

Both kistrin and echistatin prevent platelet aggregation and reocclusion in a canine model of arterial thrombolysis (63,64). Echistatin induced thrombocytopenia in baboons (65). This observation may limit the therapeutic potential of this protein, and other disintegrins.

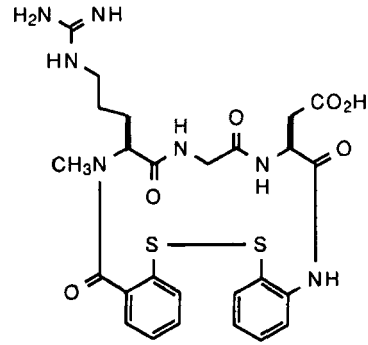
PEPTIDES

Fibrinogen Fragments- Epitopes have been identified on fibrinogen that interact with GPIIbIIIa including the RGD sequences of the *alpha* chain and the twelve C-terminal residues of the *gamma* chain (66). A mutagenesis study of the *alpha* chain RGD sequences indicated that neither epitope is sufficient to support platelet aggregation (56), however, the *gamma* chain C-terminal sequence is fundamentally important in platelet aggregation. Nevertheless, low molecular weight peptides containing the RGD sequence have been shown to inhibit platelet aggregation (67). It has been suggested that the *gamma* chain C-terminus and the *alpha* chain RGD regions share a common three-dimensional display of charged residues that explains the cross reactivity of anti-fibrinogen neutralizing monoclonal antibodies to the *alpha* and *gamma* chain fragments (42).

Early efforts in the development of RGD containing peptide antagonists of GPIIbIIIa/fibrinogen association have been reviewed (9,68,69). More recently, a cyclic disulfide, with the sequence AcCNPRGDY(OMe)RC$_{NH2}$, was reported to inhibit 100% of platelet aggregation without prolonging template bleeding time (70). A series of thioether based RGD containing peptides including *cyclo-S*-Acetyl-D-Tyr-RGDC$_{OH}$, demonstrated potent inhibition of platelet aggregation (71). A sulfoxide generated from this molecule, 1 (G4120), showed efficacy in preventing reocclusion in a canine model of thrombolysis (72) and a hamster platelet-rich femoral



1



2

vein thrombosis model (73). The diphenyl-disulfide of **2** (SK&F107260) was used to control the presentation of the RGD epitope. The structure of this material was determined by both X-ray diffraction and NMR methods (74) and has been used in the design of non-peptides (*vide infra*). An NMR structure has been reported for **1** and was also the basis for design efforts (75). The synthesis and anti-aggregatory activity of the tetrapeptide *cyclo*-RGD-L-(phenyl)Gly has also been reported (76).

GPIIbIIIa Fragments - A fibrinogen γ-chain peptide was found to crosslink in the region containing residues 294-316 of GPIIb. A peptide corresponding to residues 296-306 (TDVNGDGRHDL) from GPIIb inhibited platelet aggregation by blocking fibrinogen binding to GPIIbIIIa (77). Another peptide from GPIIb spanning residues 300-312 is also capable of inhibiting platelet aggregation by binding directly to fibrinogen (78). Furthermore, two fragments of GPIIIa are capable of inhibiting platelet aggregation. These span residues 217-231 and 211-222 of GPIIIa and also bind to fibrinogen (79,80).

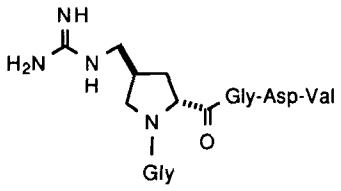
Fragments of Other Proteins - KGD containing cyclic peptides derived from the snake venom barbourin inhibit platelet aggregation with selectivity for GPIIbIIIa over other related integrins (51). The tetrapeptide KRDS from human lactotransferrin inhibits platelet aggregation induced by various stimuli presumably by inhibiting GPIIbIIIa (81). A failed attempt to prepare RGDV resulted in the tripeptide RDV which inhibits fibrinogen attachment to platelets (81). The mechanism of this inhibition may be related to that observed for KRDS. The sequence KVDL from antiflammin has been previously described as a ligand for GPIIbIIIa, but this has been discounted recently (82).

A series of hydropathically equivalent mimics of the fibrinogen binding domain on GPIIbIIIa have been designed from translation of the cRNA of von Willebrand factor and vitronectin m-RNA and reported to inhibit fibrinogen binding to platelets (83).

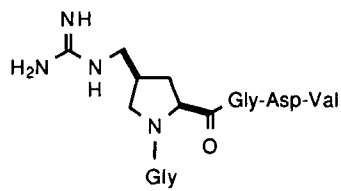
PEPTIDE HYBRIDS & NON-PEPTIDES

Unusual amino acids and non-peptidic frameworks have been used to link the C and N peptide termini, restricting the conformation of the RGD sequence. This section, however, will review various strategies taken for direct substitution of the RGD epitope with non-peptidic mimetics. Peptides that contain unnatural α-amino acids or peptide-hybrid molecules, that contain peptide backbone and side chain replacements, represent a first step in discerning the primary pharmacophoric determinants (electrostatic, shape and volume constraints) responsible for an observed activity. These replacements can provide topological and topographical information, through conformational and stereochemical restraint, supporting models of molecular recognition and binding from which the design, or selected screening, of potential lead compounds can be attempted.

Arginine replacements - Modifications of arginine of the RGD epitope have included extension of the arginine side chain to homo-arginine in linear (84,85) and cyclic peptides (86). Preparation of N-methyl arginine analogs showed an increase in potency over their corresponding arginine equivalent (87). The des-amino arginine derivatives also improved potency (87,88). A constrained presentation of the guanidine moiety using a conformationally restricted arginine side chain has been described (89). The 2R,4R isomer **3** was observed to be three times more potent than GRGDV in an Elisa assay measuring fibrinogen/GPIIbIIIa binding. This is in contrast to the 2S,4R isomer **4**, which is nearly three times less potent than GRGDV(89). Since the phenylamidine moiety



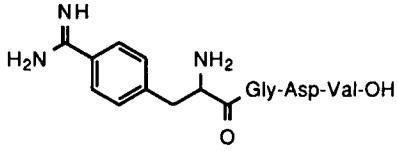
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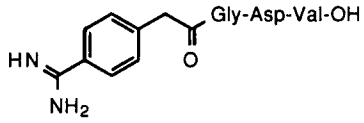
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is a useful mimic of an arginine side chain, **5** was prepared. This compound exhibited a significant increase in potency (platelet aggregation) over RGDV (90). Subsequent optimization yielded **6**. These phenylamidine derivatives are exquisitely selectivity for the integrin GPIIbIIIa over a related

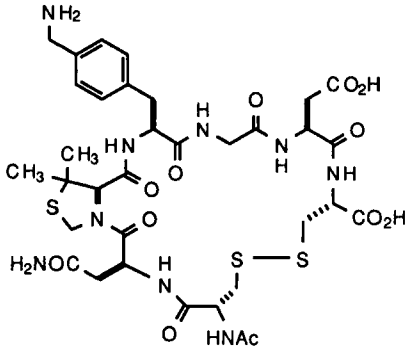
integrin, the vitronectin receptor. The potential benefit of integrin selectivity and increased potency by introduction of a phenylamidine moiety as an arginine replacement is striking (90).



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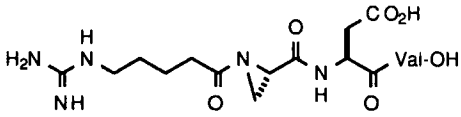
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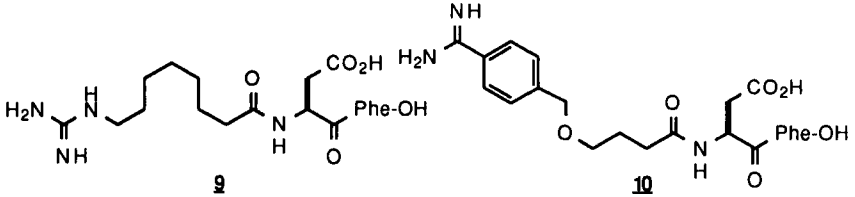
Substitution of a KGD sequence for RGD in peptides impart integrin selectivity similar to that observed for the snake venom protein barbourin (51,86). This discriminative feature was introduced into a cyclic peptide by substitution of arginine with a benzyl amine derivative, as in 7, which showed similar selectivity trends (91).

Glycine replacements - Conformational constraint of the glycine residue was achieved by introducing an aziridine moiety. The 2S enantiomer 8 was approximately 2-fold more potent than the related glycine containing peptide (92). This result is in contrast to reports indicating a loss of activity if the glycine residue is substituted with any other α-amino acid (93).



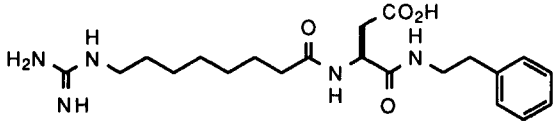
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Arg-Gly peptidomimetics - A logical progression from the des-amino arginine derivative is the replacement of the Arg-Gly amide bond as in guanidooctanoyl-Asp-X (88,94,95). For example, 9 is 25 times more potent than RGDF (96), and is further enhanced 30-fold by incorporating the phenylamidine group of 10 for the alkyl guanidine (71,97).



9 10

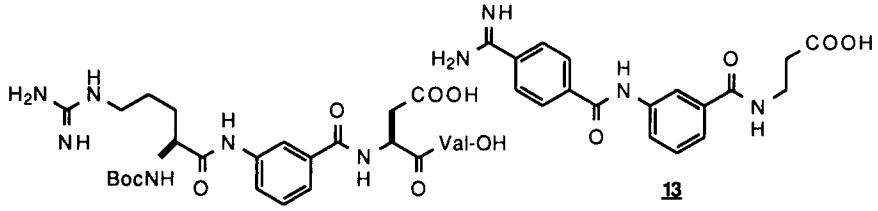
C-terminal modifications - C-terminal modifications were attempted by several groups, however, comparison of compound 9 and 11 reveal the importance of a C-terminal negative charge in binding (96). The 30-fold loss in activity observed upon deletion of the C-terminal carboxylate may indicate an intermolecular electrostatic interaction with the receptor. Alternatively, an intramolecular



11

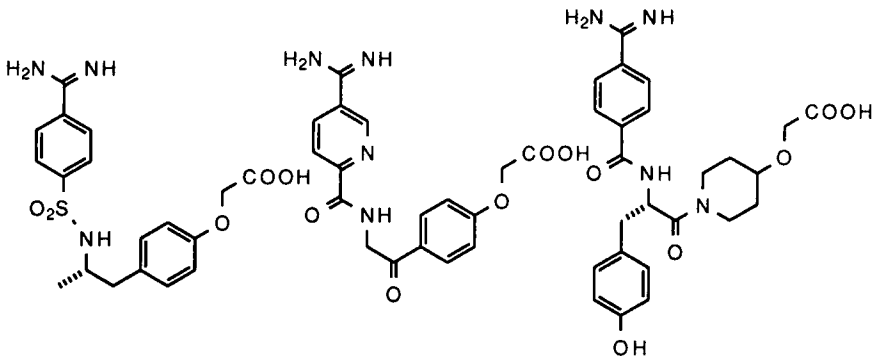
repulsive interaction between the two negative charges may induce a preferred conformation at the C-terminal end of the peptide (71). Although the C-terminal carboxylate does not appear to be fundamental to antagonist activity, its presence may have an effect on receptor selectivity or its antithrombotic profile.

Arg-Gly-Asp peptidomimetics - An alternative approach toward the replacement of the glycine residue was accomplished by taking into account apparent distance requirements between the positive and negative charges in the Arg-Gly-Asp epitope; *meta*-aminobenzoic acid in **12** was chosen as a scaffold to deliver the arginine and aspartic acid (90). Optimization of **12** by introduction of a phenylamidine for arginine and β-alanine for aspartic acid yielded **13**, one of the first reported non-peptidic GPIIbIIIa antagonists (99). The structural integrity of **13** provides unambiguous information with respect to the optimal distance between the charges as well as conformational elements of antagonism.



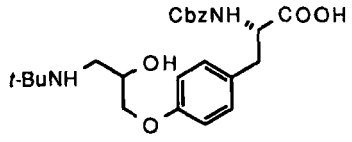
12 **13**

A previously prepared thromboxane A_2 receptor antagonist, **14**, was found through screening efforts to inhibit fibrinogen binding to GPIIbIIIa and platelet aggregation (90). Optimization resulted in preparation of **15**, a potent antagonist with high selectivity for GPIIbIIIa over vitronectin receptor (90). Again, considering the necessity for an optimal distance between the two charges that dominate this molecular interaction, a scheme was devised that benefited from ease of synthesis, utilized the potency and selectivity characteristics of the phenylamidine, and incorporated an achiral aspartic acid mimic. This led to the discovery of the tyrosine derivative **16**, (Ro44-9883, IC_{50} = 0.03μM, platelet aggregation) (90).

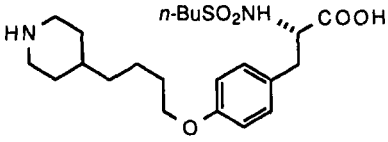


14 **15** **16**

Another tyrosine derivative **17**, was discovered *via* selected screening of a chemical library for compounds containing positive and negative charged moieties 10 to 20 Å apart (100). Iterative optimization led to **18**, which is dependent on an *alpha* amino sulfonamide, in addition to the charged moieties, for its activity. This may indicate an additional site of interaction with the receptor which may be exploited by other non-peptidic GPIIbIIIa inhibitors (100).



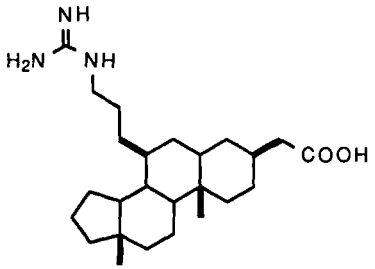
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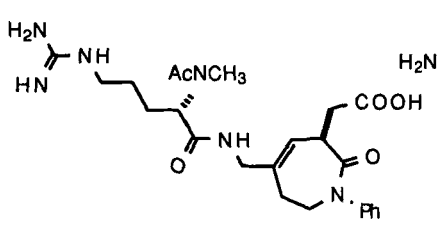
Strategies employed to take advantage of the conformational characteristics of the RGD pharmacophore (57,61,74,75,101-107) as an alternative to broad based, or directed, screening approaches to drug lead discovery have recently been reported. As a target for drug lead discovery by design, the RGD binding epitope may be ideal since it is spatially compact and its association with the GPIIbIIIa receptor is dominated by electrostatic interactions.

One study attempted to utilize the steroid nucleus to probe the conformational requirements for binding (108). Additionally, this scaffold was chosen for its generally excellent oral bioavailability and the synthetic accessibility of a variety of isomeric forms (108). Based on studies indicating that cyclic peptide *cyclo*-(GRGDSPA) includes a β-turn in which the glycine residue occupies the i+1 position (105), compound **19** was prepared. Although this compound showed relatively weak inhibition of fibrinogen binding to GPIIbIIIa, it does mark the first use of a steroid nucleus in peptidomimetic design.

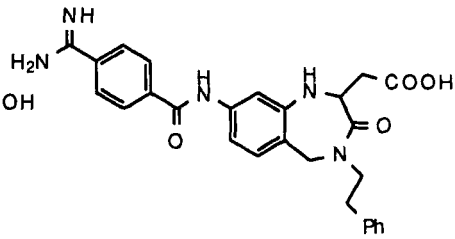


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Conformational analysis of the cyclic disulfide peptides SK&F 106760 and **2** indicated a γ-turn about the aspartic acid residue (74). The C_7 mimetic **20** was chosen for synthesis to lock this turn (110). Inhibition of Fg/GPIIbIIIa binding by **20** was observed to be slightly enhanced over SK&F 106760, supporting the hypothesis that a γ-turn about the aspartic acid residue effectively describes the biologically recognized conformer (110). Utilization of this molecular pharmacophoric model led to the discovery of compound **21**, a potent GPIIbIIIa inhibitor (110).



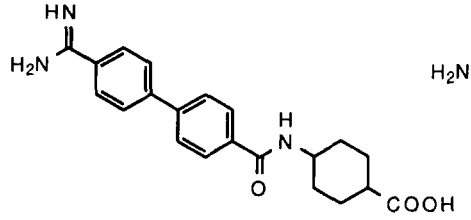
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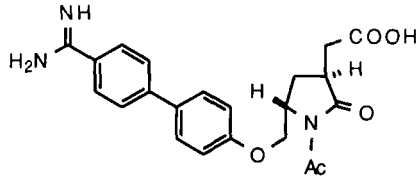
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The synthesis of non-peptide lead molecules designed to mimic a localized backbone turn was also used in the preparation of two general classes of GPIIbIIIa inhibitors based on a biphenyl motif (111). These compounds were designed from a similar model of the proposed bioactive conformation of RGDF (111). Attachment of an amidino functionality to one of the aromatic rings and tethering a carboxylate as surrogate of the aspartic acid side chain from the other aromatic ring

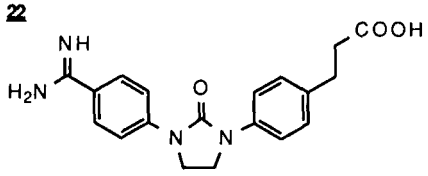
yielded **22** and **23** (112,113). Similarly, the cyclic urea derivative **24** is also a potent GPIIbIIIa inhibitor (114).



22

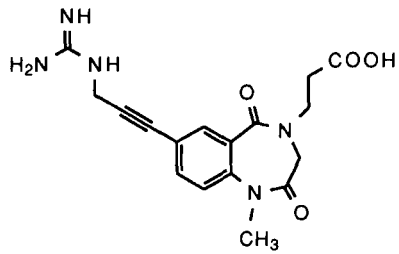


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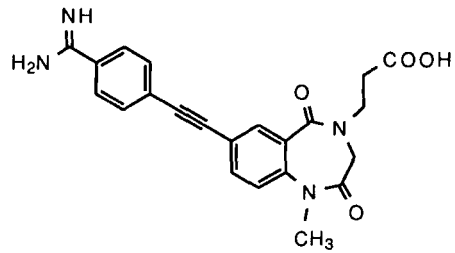


24

The design of non-peptidic GPIIbIIIa antagonists was also accomplished by mimicking the overall shape and solvophobic characteristics of **1**, the thio-ether linked cyclic peptide (75). NMR studies and molecular dynamics calculations of a series of peptides, and related analogs, identified a conformation common to each of the molecules analyzed. This consensus model, having a "cupped" shape along the edge of the RGD epitope, served as a blueprint from which compound design was initiated. The benzodiazepine nucleus was chosen for its "cupped" conformation that is consistent with the model. Thus, **25** was prepared and shown to have inhibitory properties similar to the linear GRGDV peptide (115). Optimization led to the phenylamidine derivative **26** (IC_{50} = 0.12μM, platelet aggregation), which is slightly more potent than G4120 from which it was designed (115).



25



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CONCLUSIONS

Vascular occlusion by thrombus formation has been implicated as a primary cause of a variety cerebral and cardiovascular diseases (9,116,117). Animal models of thrombosis have been devised and used to examine the biological response to GPIIbIIIa inhibition. Briefly, animal models of unstable angina by Folts (118), acute myocardial infarction (119), vessel re-occlusion after thrombolysis (45) or angioplasty (44), and stroke due to carotid arterial thrombosis (120) have been developed. The $F(ab')_2$ fragment of the anti-GPIIbIIIa monoclonal antibody 7E3 has been studied in the greatest detail followed closely by several peptide inhibitors. Reviews discussing the efficacy of GPIIbIIIa inhibition in these animal models have been reported recently (9, 121). The predictability of these models for human diseases is yet to be conclusively determined. However, clinical studies of unstable angina using $F(ab')_2$ 7E3 indicate that doses sufficient to bind 85% of the GPIIbIIIa receptors produce relief of anginal pain and complete inhibition of platelet aggregation (122). Bleeding time was prolonged to greater than 30 minutes, although no evidence of clinical hemorrhage was observed (122). As an adjunct to thrombolysis with rt-PA, the Fab fragment of 7E3 reduced thrombotic complications again without inducing excessive hemorrhage (123). Thus, it appears that an antagonist of the GPIIbIIIa/fibrinogen interaction is beneficial in the treatment of

acute human thrombotic diseases. However, the chronic use of a GPIIbIIIa antagonist will require agents that minimize bleeding events at effective doses.

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Chapter 10. Growth Factors In Cardiovascular Disease

Annette M. Doherty and Joan A. Keiser
Parke-Davis Pharmaceutical Research
Division of Warner-Lambert Company
Ann Arbor, MI 48105

Introduction – Growth factors are polypeptides that regulate the replication, differentiation and metabolic homeostasis of cells. While control of endothelial cell (EC), vascular smooth muscle cell (VSMC) and cardiac myocyte growth are poorly understood processes, abnormal growth in vascular and cardiac tissue is important in several pathologic states including hypertension, atherosclerosis and cardiac hypertrophy (1). This review will focus on individual growth factors that are implicated in the pathophysiology of cardiovascular disease.

PLATELET DERIVED GROWTH FACTOR (PDGF)

PDGF is a powerful mitogen with chemotactic activity on VSMC and a major initiator of vascular response to injury. PDGF is a homo- or hetero-dimer made of two chains (A and B) which bear 60% amino acid homology and are linked by disulfide bonds. Each of the chains are coded for on separate chromosomes and all three dimeric configurations of PDGF are mitogenic: AA, AB and BB (1). PDGF is released from activated platelets, damaged endothelium and macrophages. PDGF is under evaluation in the treatment of ulcerative disease and wound healing (2,3). It has been implicated in various other diseases including angina, obliterative bronchiolitis, venous graft occlusion, diabetic glomerulosclerosis and idiopathic pulmonary fibrosis (4-9).

Structural studies of PDGF - The crystal structure of the BB isoform of human recombinant PDGF (hrPDGF) has been determined by X-ray analysis to 3.0 Å resolution. The polypeptide chain is folded into two highly twisted antiparallel pairs of β-strands and contains an unusual knotted arrangement of three intramolecular disulfide bonds (10). Dimerization leads to the clustering of three surface loops at each end of the elongated dimer, which are thought to form the receptor recognition sites (11). Biophysical studies of PDGF have shown that the secondary structure of the molecule is composed of 54 % β-sheet with less than 5 % ordered helix (12,13).

Receptors - Two forms of the PDGF receptor (PDGF-Rα and -Rβ) have been described with differing selectivity for the three isoforms of PDGF (β is selective for BB and α binds all three isoforms). The cloning, expression and sequencing of the cDNA encoding the mouse and human PDGF-Rβ have been reported (14-16). Chimeras of PDGF-Rα and -Rß have indicated that the amino-terminal portion of the extracellular domain confers specificity for the different isoforms.

Pharmacology - In response to percutaneous transluminal coronary angioplasty (PTCA), de-endothelialization causes platelet adhesion, release of growth factors, loss of growth inhibitory factors made by the endothelial cells (e.g. heparin), and the absence of a permeability barrier to circulating aggregatory factors (i.e fibronectin) (17,18). PDGF production correlated directly with EC coverage on graft segments and inversely with platelet deposition in a canine aortic graft model (19). Continuous infusion of synthetic antithrombin to baboons after arterial injury prevented the increase in expression of PDGF-α mRNA seen in control vessels (20). Vascular lesions after angioplasty or carotid endarterectomy in baboons showed increased expression of PDGF-Rα and -Rβ in neointimal cells (21). Infusion of recombinant PDGF-BB into rats after carotid injury produced a small increase in medial VSMC proliferation. More importantly, PDGF-BB greatly increased (20-fold) vascular intimal thickening and the migration of VSMC from the media to the intima of the vessel wall (22). Expression of PDGF-Rα and -Rβ mRNA is increased in human coronary arteries after PTCA (23).

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Role in Phenotypic Modulation - Human macrophages modulate the phenotype of cultured rabbit aortic smooth muscle cells through secretion of PDGF-BB (24). Neointimal SMC from adult rats resemble a subset of medial VSMC from 12-day old rat pups and express PDGF-Rβ gene but have little or no PDGF-Rα mRNA (25). In contrast, medial SMCs from uninjured vessels contain abundant PDGF-Rα mRNA but little or no PDGF-Rβ mRNA. The differences in VSMC phenotypes observed in cell culture may depend on unique conditions; analogous VSMC diversity is not known *in vivo* (25).

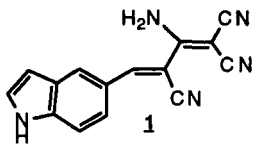
Signal Transduction - In response to binding, the PDGF-Rβ subunit is phosphorylated on tyrosine (Tyr) residues and associates with various signal transduction enzymes, including the GTPase-activating protein of ras (GAP), PLC-γ and phosphatidylinositol 3-kinase (PI3K) (26). Cloning of the 85-kDa subunit of PI3K (p85) and the solution structure by NMR spectroscopy were recently reported (27, 28). Site directed mutagenesis indicates that Tyr740 and 751 are involved in PDGF-stimulated binding of PI3K, important for mitogenic activity, and Tyr771 is required for efficient binding of GAP, while Tyr1021 and possibly Tyr1009 represent the binding site for PLC-γ (29, 30). PDGF Tyr phosphorylation of PLC-γ is not thought to be required for mitogenesis and transformation (31). Chimeric receptors composed of extracellular domain of human epidermal growth factor receptor were fused to wild type and truncated PDGF-R intracellular sequences. These chimeric receptor experiments implied that 80 C-terminal residues of the PDGF-R (possibly Tyr-989) in the PLC-γ binding sequence upstream from Asp-988 were essential for specific interactions with GAP and PI3K (32). Complexation of a 17-residue peptide surrounding phosphorylated Tyr751 with the SH2 domain of PI3K has been reported (33).

The extracellular glycoprotein SPARC inhibits the binding of PDGF-AB and -BB but not PDGF-AA, to human dermal fibroblasts. Enhanced expression of PDGF-B chain and SPARC is seen in advanced atherosclerotic lesions (34). PDGF-A[108-124] competes with PDGF for binding to heparin. The sequence [-Lys115-Lys116-Arg117-Lys118-Arg119-] is important in the specific binding between PDGF and heparin (35). PDGF stimulates the expression of genes associated with fibroblast cell cycle entry, including c-*myc* and c-*fos* (36). JE mRNA, a PDGF-inducible "early" gene, accumulates rapidly in aortic injury and in PDGF-stimulated VSMC (37).

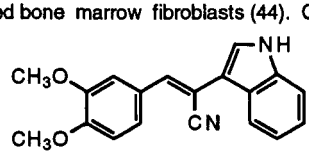
Peptide Antagonists - A peptide containing 12 amino acids from the N-terminal and 17 amino acids from the C-terminal region of PDGF-B binds to PDGF-Rα and -Rβ (IC_{50} = 6μM). A PDGF-B chain peptide with residues [116-121] and [157-163], ANFLVWEIVRKKP, was reported as an antagonist at both receptors (38). In the A-chain, antagonists from the region containing residues 80 and 110 at the C-terminus have been reported (39). PDGF-A [194-211] binds specifically, saturably and with low affinity to a large population of sites on Balb/c 3T3 and several other cell lines (40). This peptide modulated the cellular proliferative response to normal human serum and several polypeptide growth factors, likely due to an interaction with extracellular glycosaminoglycan (40).

PDGF analogs where the 2nd and 4th cysteine residues were mutated to serines have been reported as agonists (41). PDGF-B chain analogs resistant to proteolysis have been reported (42). A single amino acid exchange in domain II of PDGF-AA (Ala67 --> Arg) reduced binding to the β-receptor. In domain I exchanging residues Pro26 --> Arg and Ser28 --> Asn changed the specificity towards the β-receptor, indicating that parts of these domains are involved in receptor binding (43).

Other Modulators of PDGF Action - Compound **1** (AG370) and tyrphostins inhibited PDGF-induced mitogenesis and tyrosine kinase (TK) activity in cultured bone marrow fibroblasts (44). Compound **2**

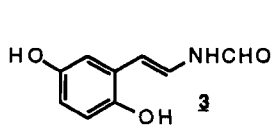


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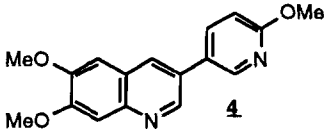


2

(the tyrphostin RG-13291) blocked PDGF-Rβ PI3K association in VSMC (45). PDGF TK activity is enhanced in injured carotid arteries and is inhibited *in vivo* by **2** (46). Compound **3** (erbstatin) inhibits PDGF and EGF TK activity (47). The pyridylquinoline derivative **4** inhibits PDGF-R autophosphorylation (IC_{50} =30 to 70 nM)(48).

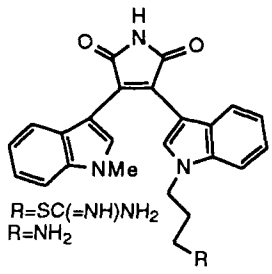


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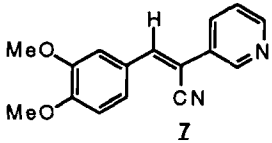


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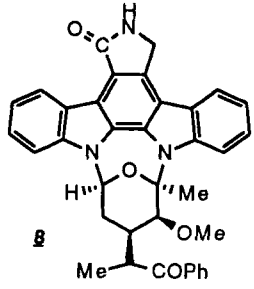
Compounds **5** (RO-318220) and **6** (RO-317549) are potent inhibitors of the proliferation of cultured rabbit aortic SMC in response to fetal calf serum, serotonin and PDGF-BB, or phorboldibutyrate (49). Compound **7** (RG-13022) inhibits the activity of several growth factors (50). Compound **8** (CGP 41251, N-benzoylstaurosporine) directly inhibits PDGF-R receptor autophosphorylation and *c-fos* mRNA expression induced by PDGF stimulation of intact BALB/c 3T3 cells (51). Several phosphorylated pentapeptides inhibit the p85-PDGF-Rβ association i.e. Tyr(PO_3H)–Val–Pro–Met–Leu (52). Neomycin specifically inhibits the autophosphorylation of the α-receptor by PDGF-BB (53). There have been several excellent reviews covering TK inhibitors (54).



5 R=SC(=NH)NH_2
6 R=NH_2

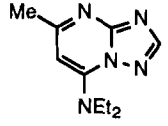


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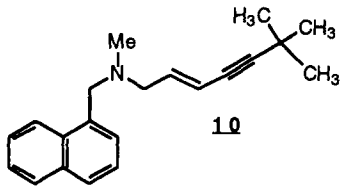


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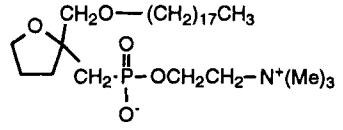
A wide range of agents, some of whose mechanism of action is unclear, appear to inhibit the mitogenic and/or migratory effects of PDGF. Compound **9** (trapidil) inhibited proliferation of PDGF-associated growth in human glioma cells, causing morphological changes and increases in glial fibrillary acidic protein content (55). Trapidil reduced the incidence and progression of restenosis and chest pain in a randomized trial in patients after PTCA (56). Side-effects included skin eruption, raised aspartate aminotransferase and glutamic-pyruvic transaminase levels. The thiol protease inhibitor (Ac-Leu-Leu-Norleucinal, *TPI-1*) inhibited *PDGF-BB* stimulated *DNA* synthesis with an EC_{50} of 3μM (57). Other agents reported to functionally inhibit PDGF include **10** (terbinafine) (58), staurosporine (51), **11** (SRI 62834) (59), **12** (octreotide) (60), simvastatin, lovastatin and nifedipine



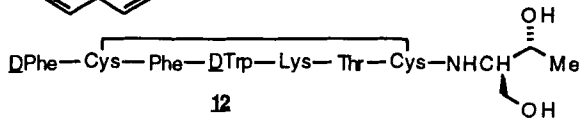
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11



12

(61), ticlopidine (62), elcosapentaenoic acid, trieicosapentaenoyl glycerol (63), colchicine (64), hexamethylenebisacetamide (65), retinoids (66), several calcium antagonists (67), ginsenosides (68) and indapamide (69). Inhibitors of ornithine decarboxylase, including α-difluoromethylornithine and various aminochromones inhibits PDGF-promoted growth (70, 71).

FIBROBLAST GROWTH FACTORS (FGFs)

Introduction - Fibroblast growth factors (FGFs) are a family of structurally related polypeptides; seven members have been identified to date. Acidic and basic FGF (aFGF and bFGF) are the best characterized members of these heparin-binding proteins. FGFs are broad spectrum mitogens and their role in cardiac and VSMC has been reviewed (72, 73). Systemic administration of aFGF or bFGF produces hypotension in rodents by unknown mechanisms (74). Stabilized analogs of FGF have been claimed in recent patents for use in angiogenesis, ischemia and wound healing (75, 76).

Receptors - Multiple receptors for FGF have been described, most recently a cysteine rich receptor was cloned, expressed and characterized (77). Mutations of an FGF receptor have successfully separated the mitogenic and calcium mobilizing effects of FGF, suggesting distinct signalling pathways (78, 79). The signal transduction pathway for FGF TKs has been reviewed (80). Four tyrosine residues have been identified as phosphorylation sites in the TK isoform of the FGF-1 receptor (81). TK activity alone is insufficient to account for the full spectrum of biological activities of bFGF (82). Although the intracellular fate of bFGF is unknown, FGF can be internalized through direct interaction with cell surface heparan-sulfates (i.e. non-receptor mediated internalization) (83).

Pharmacology - Systemic administration of exogenous bFGF stimulates angiogenesis in rats and rabbits after severe hindlimb ischemia (84, 85). In addition, bFGF reduces infarct size and increases the number of arterioles and capillaries in a canine infarct model (86). Investigators have reported no change in the expression of bFGF after vascular injury (angioplasty); although FGF receptors are upregulated (87). Transfection of a gene encoding secreted FGF-1 into porcine carotid arteries *in vivo* promotes intimal hyperplasia and angiogenesis (88). It has been suggested that heparin inhibits VSMC proliferation in response to vascular injury by depleting released bFGF from sites of injury (89).

Chemically modified heparins potentiate the mitogenic effects of aFGF; however there is no correlation between mitogenic activity and binding of the heparins to FGF (90, 91). Antibodies raised to bFGF inhibited VSMC proliferation, but not lesion size in a rodent model of restenosis (92). Similarly, a recombinant toxin targeted to the FGF receptor, blocked VSMC proliferation (93); truncated and mutated FGF receptors also inhibited the biological actions of bFGF *in vitro* (94, 95). Antisense oligonucleotides to bFGF inhibited expression and EC proliferation *in vitro* (96).

Antagonists - The glycoprotein, SPARC, antagonizes the mitogenic effects of bFGF (97). Suramin inhibits angiogenesis through blockade of bFGF activity, possibly by producing microaggregation of the growth factor (98, 99). The peptides X'_n–RSRK–X_n (where X and X' are independently any amino acid) and RKLAV–YWSSY–KRSRY are reported to have FGF antagonist properties (100 , 101). In addition, truncated FGF analogs (12 amino acids deleted at the N-terminus and 20 at the C-terminus) are claimed as FGF antagonists (102). FGF fragments consisting of residues 27-69 and/or amino acids 70-155 are reported to act as FGF agonists with improved stability compared to native FGF (103).

TRANSFORMING GROWTH FACTOR-β (TGF-β)

TGF-β1 is a homodimeric 25kD polypeptide secreted by most mammalian cells in latent form (104, 105). There are four closely related isoforms in vertebrates and three in man (β1,2,3). TGF-β acts as a stimulator and inhibitor of cell replication, involved in the regulation of differentiation, cell adhesion and extracellular matrix deposition (104, 105). Recombinant TGF-β is being studied for treatment of inflammatory disorders and in wound healing (106, 107). Antagonists to TGF-β may be of utility in progressive glomerulosclerosis and cancer (108). Antibodies to TGF-β reduced the number of macrophages and amount of collagen in rat wounds, indicating utility in the control of scarring (106, 108). The role of TGF-β in human disease has recently been reviewed (109).

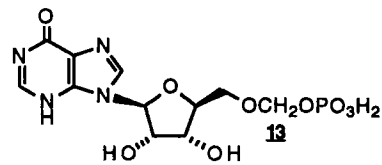
Structure - X-ray crystallography of the TGF-β2 dimer, determined at high resolution, indicated an extended structure (110). NMR analysis of TGF-β1 in solution has also recently been reported (111).

Receptors - Five types of TGF-β receptor have been identified: types I and II that are ubiquitous have been cloned and are involved in mediating the biological activities of this growth modulator (112). The recently cloned type II receptor is a functional serine/threonine kinase and the type V receptor also has serine/threonine-specific autophosphorylation activity (113-115). Endoglin, the type III binding site, has not been shown to be directly involved in signal transduction (116). Endoglin is found in high concentrations in endothelial cells and binds TGF-β1 and β3 with high affinity, while it does not bind TGF-β2 (116).

Cardiac Effects - Myocardial stunning stimulates TGF-β expression that may be important in collateral development (117). It inhibits TNF release into the blood after myocardial infarction and may be involved in the regulation of cardiomyocyte proliferation and differentiation (118). An antibody, effective against several TGF-βs, prevented myocardial cell transformation (119). TGF-β1 attenuates neutrophil adherence to ischemic-reperfused cardiac myocytes and may protect the heart from reperfusion injury (120). Increased mRNA for matrix proteins is preceded by increases in TGF-β1 mRNA in cardiac hypertrophy (121).

Vascular Effects - Expression of TGF-β is increased in human vascular restenosis lesions and in aortic SMC from spontaneously hypertensive rats (SHR) (122, 123). AII is reported to increase TGF-β1 gene expression via a protein kinase C dependent pathway, and promotes the conversion of latent TGF-β1 to its biologically active form (124). TGF-β1 inhibited VSMC proliferation stimulated by PDGF-BB in Wistar-Kyoto (WKY) rats (125). TGF-β regulates increased ductus arteriosus endothelial glycosaminoglycan synthesis associated with intimal proliferation (126). Its synthesis is correlated with EC density and cell spreading, implicating TGF-β in the development of atherosclerotic lesions (127). TGF-β inhibits the production of inducible nitric oxide in VSMC (128). It also stimulates the synthesis and release of adrenal renin and prorenin and inhibits aldosterone production in cultured bovine zona glomerulosa cells (129).

Modulators of TGF-β - Suramin partially reversed TGF-β induced growth inhibition in cultured human renal cell carcinoma (130). Recombinant human decorin, a proteoglycan that may naturally modulate TGF-β, prevented extracellular matrix deposition in injured glomeruli from nephritic rats and reduced TGF-β production *in vivo* (131). Fucoidan (a polymer of L-fucose monosulfate) and polyinosinic acid (a polymer of 5'-inosinic acid, **13**) bound to PDGF and TGF-β_1 receptors and inhibited proliferation of rat aortic SMC *in vitro* (132). The antiproliferative effects TGF-β_1 on EC were modified by cysteine, cystine, and N-acetylcysteine with no evidence for direct inactivation of TGF-β_1 (133). Residues 40- 82 of TGF-β [40-82] are important for specificity of TGF-β_1 vs. -β_2 (134). Tamoxifen induces TGF-β in the stroma of human breast tumours (135, 136).



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VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

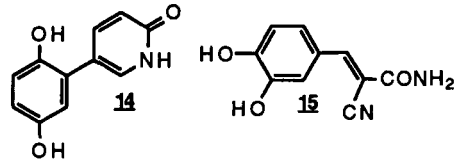
VEGF, also called vascular permeability factor, was identified and purified from rat glioma cells; the purified protein is a 46 kDa dimer composed of two subunits (137). VEGF is a potent angiogenic factor specific for EC (138). Expression of VEGF is increased in porcine myocardium that has undergone collateralization (139). Hypoxia induces VEGF expression and may be important in angiogenesis in response to hypoxia; inhibition of VEGF-induced angiogenesis inhibits tumour growth *in vivo* (140, 141). Coronary collateral blood flow is increased after intracoronary VEGF administration (142). The VEGF receptor has TK activity; expression of *fms*-like TK complementary cDNA in COS cells conferred specific, high affinity binding of VEGF (143). A recent patent has appeared claiming cloned VEGF for control of vascular growth (144).

OTHER GROWTH FACTORS

Epidermal Growth Factor (EGF) - Human EGF is a 53 amino acid peptide with three disulfide bonds and the sequence is well conserved across species. The EGF receptor was cloned and sequenced in 1984 (145). EGF is known to play a role in epithelial cell growth, however its effects on vascular and cardiac tissue are less clear. EGF receptors are upregulated in aortic tissue of rats chronically

exposed to pressor levels of AII (146). In human coronary VSMC from restenotic lesions, the proliferative effects of EGF were enhanced compared to cells from primary atherosclerosis, suggesting that EGF may play a role in intimal hyperplasia post-angioplasty (147). A recombinant fusion protein specific for the EGF receptor inhibited VSMC growth in human atherosclerotic plaques *in vitro* (148). EGF receptors are upregulated in the kidney and aorta of genetically hypertensive rats (149). EGF also promotes kidney growth acutely; however EGF treatment had no effect on the progression of chronic renal failure in rats following subtotal nephrectomy (150).

The pyridone **14** (RG 14921) inhibits EGF receptor TK activity non-competitively (151). Inhibitor **15** and several aminoalkyl-acrylophenones also selectively block EGF receptor activity (152-154).



Insulin-like Growth Factor (IGF) - IGF-I, also known as somatomedin-C (SM-C), is a basic single chain polypeptide of 70 amino acids containing three disulfide bonds. IGF-II is an acidic peptide containing 67 amino acids (155). IGF-II is 60% homologous with IGF-I; both peptides have a high structural homology with human proinsulin. The IGF peptides have three characteristic activities: stimulation of glycosaminoglycan sulfation, insulin-like activity, and mitogenic activity. IGF receptors have been characterized in adult rat cardiac myocytes (156). IGF treatment stimulates cardiac protein synthesis (157). In the post-ischemic rat kidney undergoing regeneration, IGF-I binding and synthesis are increased, suggesting that IGF-I is a local trophic agent (158). IGF-I increases glycosaminoglycan production by renal mesangial cells in the glomerulus and may be linked to renal hypertrophy in diabetes (159).

Nerve Growth Factor (NGF) - NGF exists as a complex of three subunits with a molecular weight of approximately 130,000 (160). NGF mRNA is elevated in resistence vessels of young SHR (161). In addition, sciatic nerve and spleen NGF content was elevated in young SHR compared to WKY controls suggesting a role for NGF in enhancing peripheral sympathetic nervous system activity and overproduction of vasoconstrictor catecholamines in these hypertensive animals (162). However, newborn WKY rats treated with NGF for 2 weeks failed to develop hypertension despite elevated adrenal, brain and mesenteric artery levels of norepinephrine (163).

Macrophage Colony Stimulating Factor (M-CSF) - The cytokine M-CSF, also known as CSF-1, was first isolated from cultured mouse L cells as a heavily glycosylated 85 kDa homodimeric protein (164). The cDNA for human M-CSF was reported in 1985 (165). The structure of human recombinant M-CSF has been determined by X-ray crystallography (166). M-CSF specifically stimulates monocyte-macrophage function and may play a role in cholesterol metabolism of macrophages. M-CSF treatment enhanced low density lipoprotein uptake in macrophages and efflux of cholesterol from cholesterol-loaded macrophages (167). M-CSF treatment retarded atherosclerotic progression in Watanabe hyperlipidemic rabbits (168).

ANTAGONISM OF INTRACELLULAR SIGNALING – NEW APPROACHES TO INTERVENTION

Several oncogenes have been suggested to play a role in VSMC proliferation. Treatment of SMC with antisense c-*myb* phosphorothiolate oligonucleotides inhibits SMC proliferation *in vitro* and intimal arterial SMC accumulation *in vivo* after rat carotid artery balloon-injury (169, 170). Antisense oligonucleotides to c-*myc* dose-dependently inhibited VSMC proliferation and migration *in vitro* (171). Proliferating cell nuclear antigen (PCNA) is an intranuclear protein that functions in a final common pathway shared by diverse mitogen-induced signals. An antisense oligonucleotide targeting PCNA mRNA decreases the expression of PCNA gene product and reduces SMC proliferation *in vitro* (172).

FUTURE DIRECTIONS

Growth factors can stimulate and in some cases inhibit cellular proliferation and can also act as chemoattractants. Cell proliferation and migration are critical events in the development of atherosclerotic lesions and in the restenosis that can occur following coronary bypass surgery,

endarterectomy and balloon angioplasty. Interactions among the growth factors in their release and actions are well documented. Antagonists to the various growth-regulatory polypeptides either at the receptor level or by intervention of their signalling pathways may provide novel treatments for the therapy of a variety of vascular proliferative disorders.

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Chapter 11. The Role of Tachykinins in Pulmonary Disease

John A. Lowe, III and R. Michael Snider
Central Research Division, Pfizer Inc
Groton, CT 06340

Introduction — The neuropeptide substance P (SP) is widely distributed in the central and peripheral nervous systems where it has excitatory effects on neurons and is a principal neurotransmitter of the polymodal nociceptive afferent neurons (C-fibers). Its distribution, biosynthesis, cellular mechanism of action, and biological effects have been reviewed (1,2). The biological effects of SP on non-neuronal tissues, which result directly from SP release following C-fiber activation, include stimulation of smooth muscle contraction, exocrine and endocrine gland secretion, and endothelial cell stimulation leading to vasodilation, plasma extravasation, and neutrophil infiltration. These latter, peripheral actions of SP are commonly termed neurogenic inflammation and this review will focus on the possible role of SP and related peptides in airway inflammatory conditions commonly known as asthma, bronchitis and rhinitis.

Figure 1
Primary Structure of Substance P and Related Mammalian Tachykinin

Peptide	*Sequence*
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Neuropeptide K	Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	His-Ser-Ile-Gln-Gly-His-Gly-Tyr-Leu-Ala-Lys
	Asp-Ser-Ser-Ile -Glu-Lys-Gln-Val-Ala-Leu-Leu
	Ala-Asp
Neuropeptide γ	Lys-Arg-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
	His-Ser-Ile-Gln-Gly-His-Gly-Ala-Asp

The Tachykinin Peptides and Their Receptors — The mammalian tachykinin (which literally means fast-acting) peptides include SP, neurokinin A and B (NKA, NKB), and the N-terminally extended forms of NKA which include neuropeptide K (NPK) and neuropeptide γ (NPγ) (Figure 1). Agonist activity is defined by the shared carboxy terminal domain (Phe-X-Gly-Leu-Met-NH_2), whereas the amino terminal domains determine receptor binding selectivity. These mammalian tachykinin polypeptides are derived from two distinct genes, the SP/NKA gene and the NKB gene (3,4). There are three mRNA which are produced from the SP/NKA gene as a result of alternative RNA splicing, termed α-, β-, and γ-preprotachykinin (PPT) mRNA. While α-PPT can encode only SP, β- and γ-PPT mRNA can encode both SP and NKA. Further post-translational processing of the β-PPT mRNA product can yield NKA or NPK, while γ-PPT mRNA can produce NKA or NPγ (5,6,7). NKB is the only tachykinin peptide produced from the NKB gene. These several tachykinin genes, with their multiple RNA splicing and post-translational processing mechanisms, generate substantial diversity in the tachykinin peptides which can be produced.

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Table 1. Mammalian Tachykinin Receptors

Receptor Type	*Ligand Specificity*	*Pulmonary Localization*	*Biological Correlate*
NK_1	SP>NKA>NKB	- endothelial cell	- vasodilation - capillary permeability - edema
		- submucosal gland	- mucus secretion
		- epithelial cell	- EDRF, PGE_2 production
NK_2	NKA>NKB>SP	- airway smooth muscle	- contraction and bronchoconstriction
NK_3	NKB>NKA>SP	- none identified	

The three mammalian tachykinin receptors (Table 1) have been cloned and shown to be members of the G protein-coupled receptor family (8). They are coupled to inositol phospholipid metabolism and a rise in the cytosolic free calcium concentration (9). Each of the three receptors, which are termed neurokinin-1 (NK_1), NK_2, and NK_3, displays a moderate degree of selectivity for SP, NKA (also NPK and $NP\gamma$), and NKB, respectively. However, if present at sufficiently high concentrations, each of the tachykinin peptides is fully capable of eliciting responses from all three receptors, presumably due to the shared sequence homology of the carboxy terminus. This overlapping specificity of agonist activity may be particularly important in pulmonary functions of tachykinins since NPK and $NP\gamma$ are exceptionally potent at both the NK_1 and NK_2 receptor types.

ANATOMICAL LOCALIZATION AND FUNCTION

Peptides — SP- and NKA-immunoreactivity has been demonstrated in the neuronal innervation of the airways in many mammalian species, including man (10). C-fiber terminal varicosities are found around airway smooth muscle, epithelium, submucosal glands and blood vessels. The ability of capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent principle of plants of the *Capsicum* family, to release and subsequently deplete SP from these nerve endings (11-16) has been pivotal in understanding the distribution and physiological roles of the tachykinins. Subsequent work by many laboratories has established the functions of tachykinin peptides released by capsaicin in the nasal mucosa and lung mediating enhanced vascular permeability, vasodilation, bronchoconstriction and submucosal gland secretion (for reviews see 17,18).

The vagal nerves contain afferent SP-immunoreactive nerve fibers from the lung (19). The cell bodies of these afferent C-fibers in the dorsal root ganglia (nodose ganglion) produce SP, 90% of which is transported into peripheral vagal branches (20). This implicates SP as the transmitter of nociceptive information to the CNS (21) and at the peripheral terminal (14). Thus, upon sensory nerve stimulation, the action potential is conveyed to the spinal cord and also depolarizes the extensively branched peripheral collaterals. This axon reflex results in the release of transmitter peptides over the entire anatomical distribution of the nerve and may serve to amplify the intitiating stimulus. Capsaicin-sensitive C-fibers are also known as polymodal nociceptive afferents, which means they are activated by thermal, mechanical and chemical stimuli at their nerve endings. This is a key observation regarding the possible role of tachykinin-induced neurogenic inflammation in asthma and bronchitis as these diseases are often initiated or exacerbated by inhaled irritants. In addition to SP, C-fibers also contain NKA, NPK, and possibly $NP\gamma$, all very potent agonists at NK_2 receptors, which can be coreleased in the lung upon afferent stimulation (22).

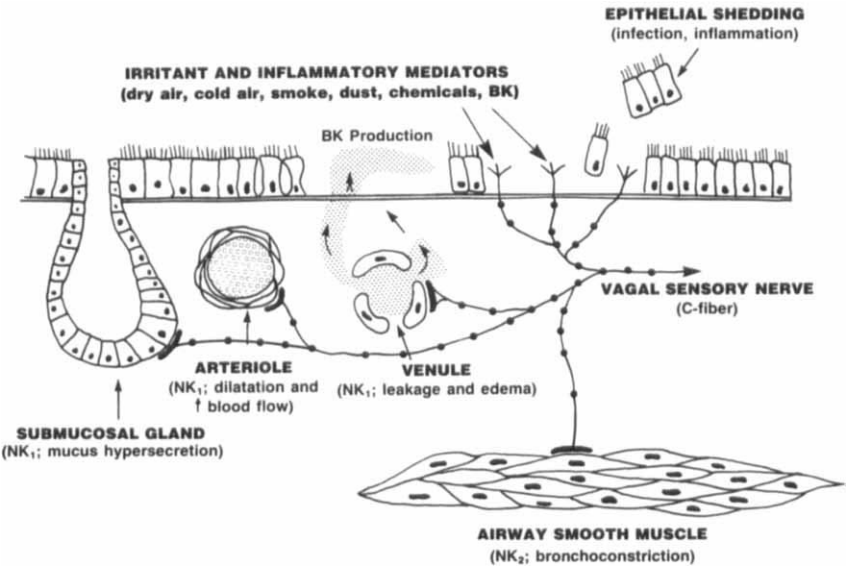
Receptors — Pulmonary distribution of tachykinin receptors, the associated ligand specificity, and biological actions of receptor stimulation are summarized in Figure 2. Tachykinin receptors have been visualized autoradiographically with radiolabeled SP in rat, guinea pig, rabbit, and human lung (23-26), although specific labeling of receptor subtypes using selective radioligands has not been reported.

The NK_1 receptor has an extensive pulmonary distribution, and is localized on endothelial cells, epithelial cells, exocrine glands and several types of circulating leukocytes, suggesting a role in neurogenic inflammation. The NK_2 receptor is found at highest density on airway smooth muscle cells in the lung where it may effect bronchoconstriction. The NK_2 receptor is also found on neurons in the parasympathetic autonomic ganglia and on postganglionic neurons and facilitates pulmonary cholinergic transmission (27-29). There are also NK_2 receptors in the brain and spinal cord with a pattern of distribution that is nonoverlapping with that of the NK_1 receptor. Finally, while the NK_3 receptor is found (depending on species) in both the CNS and in the periphery, notably in the gastrointestinal tract and in vascular smooth muscle, it has yet to be identified in pulmonary tissues.

Tachykinin Metabolism — Endogenous tachykinins are degraded by two principle enzymes: angiotensin-converting enzyme (ACE, EC 3.14.5.1) and neutral endopeptidase (NEP, EC 3.4.24.11), also called enkephalinase (30). NEP is localized in basal cells of the epithelium, postcapillary venules, sensory nerves and submucosal glands and is likely the primary route of tachykinin metabolism in lung tissue (31). ACE is localized primarily on endothelial cells where it may mediate intravascular tachykinin catabolism. Its potential importance in pulmonary disease is emphasized by the finding that ACE inhibitors enhance the broncoconstrictor response to SP in humans (32).

TACHYKININS IN PULMONARY DISEASE

Asthma and bronchitis — Asthma is mediated by a complex interplay of multiple factors, but the mechanisms responsible for its symptoms of reversible bronchoconstriction, mucus hyperproduction, cough, and underlying inflammation have yet to be defined. Nonetheless, there is extensive evidence for involvement of tachykinins in the pathophysiology of asthma. Asthmatics have increased immunoreactive SP (33) and NK_1 receptor gene expression (34) compared with nonasthmatic controls. Moreover, inhaled NKA causes significant bronchoconstriction in asthmatics (35) and tachykinins enhance mucus secretion from human isolated bronchi (36), raising the possibility that nociceptive or irritant-induced release of tachykinins from sensory C-fibers via the axon reflex (Figure 2) contributes to the pathophysiology of asthma (37-40). In addition, pretreatment with capsaicin has been shown to induce airway hypersensitivity to nonspecific stimuli, suggesting that endogenous tachykinins may mediate asthmatic hyperresponsiveness (41). Inhibition of NEP with thiorphan or phosphoramidon potentiates plasma extravasation induced by exogenous tachykinins (42,43) or hypertonic saline (44). NEP inhibition also potentiates bronchoconstriction (45-48), cough (49), and mucus secretion (50) in humans. Conversely, exogenously administered NEP has been shown to attenuate SP- or capsaicin-induced plasma protein extravasation and cough (51,52).

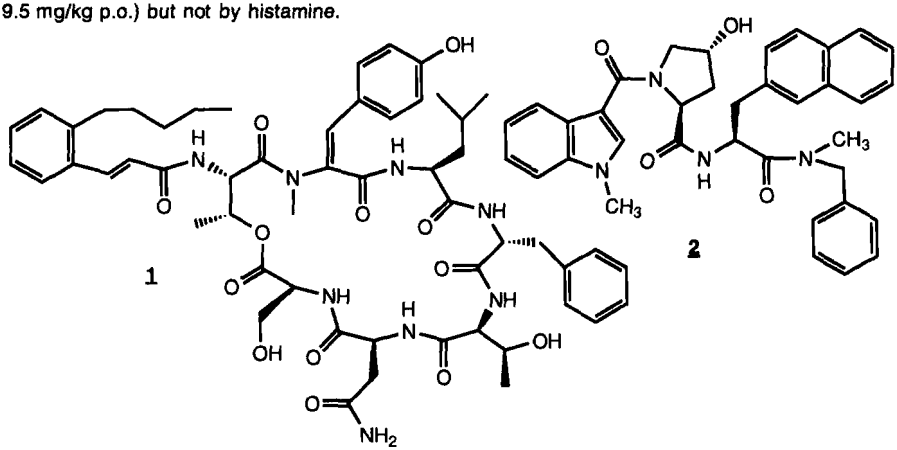


Many other aspects of pulmonary disease suggest the involvement of tachykinins. Viral respiratory infections (53-56), cigarette smoke, or other chemical irritants (57-59) markedly enhance the neurogenic inflammation induced by tachykinins. Viral infection or airway inflammation leads to a shedding of bronchial epithelium (Figure 2), including NEP-containing cells, allowing easier access of irritants to C-fiber nerve endings, and enhancing penetration of allergens to inflammatory cells in the submucosa (60). Upon depolarization of the afferent sensory C-fibers, tachykinin peptides contained in the varicosities of the fiber are released locally as well as in the dorsal horn of the spinal cord. Locally released peptides acting via NK_1 and NK_2 receptors are capable of causing bronchoconstriction, increased blood flow, plasma extravasation and the resultant extraluminal edema which leads to further closing of airways (61), mucus hypersecretion, and cough. Finally, glucocorticoids, a widely used treatment for asthma, have been shown to reduce SP- or capsaicin-induced plasma extravasation (62), expression of NK_1 receptor mRNA (63,64), and the SP content of sensory neurons (65).

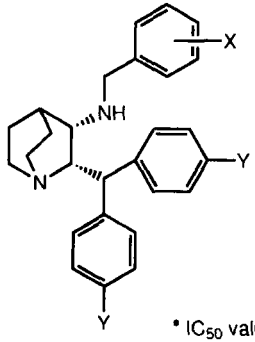
Rhinitis — As opposed to the effects in lower airways, the role of sensory neuropepides within human nasal mucosa is less well defined, but the possible involvement of sensory neuropeptides has been recently reviewed (66,67). Immunoreactive SP and NKA have been identified in human nasal mucosa nerve fibers around arterioles, venules, sinusoids, submucosal glands, and basement membrane epithelium (68). Similarly, the distribution of autoradiographically localized binding sites for radiolabeled SP and NKA is consistent with a role of sensory tachykinins in regulating blood flow, vascular permeability and mucus secretion. Intranasal capsaicin causes rhinorrhea, pain and sneezing in normal humans (69), and exaggerated responses in patients with vasomotor rhinitis (70). In patients with allergic rhinitis, however, intranasal SP produces nasal obstruction and vascular leakage (71) while a much weaker response is observed in normals (72). Clinical evidence for the involvement of tachykinins in rhinitis comes from recent reports of a treatment regimen of repeated applications of intranasal capsaicin in patients with vasomotor rhinitis with significant improvement in symptoms reported for a 1 to 6 month follow-up period (73,74).

TACHYKININ ANTAGONISTS

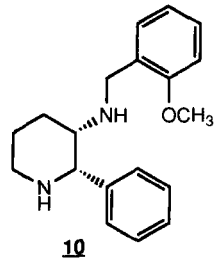
Peptide and non-peptide tachykinin antagonists — Early efforts to find a SP antagonist focused on replacement of key amino acids in the sequence of SP with D-amino acids, resulting in the discovery of spantide (IC_{50} = 80 nM) (75) and spantide II (IC_{50} = 20 nM) (76). Another approach, broad-based natural products screening, led to the cyclic polypeptide FK224 (1) prepared by reduction of the fermentation- derived WS-9326A (77). 1 displays a mixed spectrum of tachykinin antagonism, with IC_{50} values of 37 nM and 72 nM at the guinea pig NK_1 and rat NK_2 receptors respectively (78). Yet another peptide-based approach, systematic exploration of peptidic SAR, began with an octapeptide SP antagonist, elucidated the tripeptide core responsible for activity (79), then modified it to a dipeptide, FK888 (2) using a hydroxyproline core for stability and solubility (80). 2 shows IC_{50} values for displacement of [^{3}H]-SP of 6.9 nM in guinea pig lung and 1.8 μM in rat brain (81). In addition, it blocks airway edema in the guinea pig induced by SP (ED_{50} = 4.2 mg/kg p.o.) and capsaicin (ED_{50} = 9.5 mg/kg p.o.) but not by histamine.



A completely different strategy in this area involves discovery of nonpeptide SP antagonists based on empirically directed compound screening. The first nonpeptide SP antagonist CP-96,345 (2*S*,3*S*-**3**) was the result of an extensive chemical synthesis program following up on a lead quinuclidine series uncovered by directed screening (82). SAR results showing the effect of substitution on the benzylamine side chain, (**4**) to (**8**), and the benzhydryl group (**9**) are summarized below (83). Consideration of these two elements along with the bridgehead nitrogen, which is critical for receptor recognition, led to a three-point binding model for the interaction of 2*S*,3*S*-**3** with the NK_1 receptor. 2*S*,3*S*-**3** shows IC_{50} values of 3.4 nM and 0.77 nM at the NK_1 receptor in bovine caudate and human IM-9 cells respectively. In addition, it was found to block plasma protein extravasation induced by SP or mustard oil in the rat, in the latter case with an ED_{50} value of 4 mg/kg p.o. (84). 2*S*,3*S*-**3** was also effective in the acetic acid-induced writhing model in the mouse, with an ED_{50} value of 10.7 mg/kg p.o., indicating its potential antiinflammatory and analgesic activity (85). The disclosure of a piperidine with increased potency in binding to the NK_1 receptor CP-99,994 (**10**) indicates extensive opportunity for further research in this area (86).



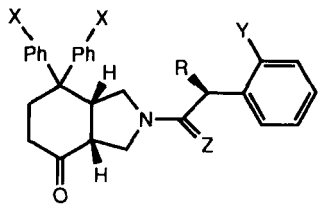
	X	Y	SP IC_{50}*
3	2-OCH_3	H	2.2
4	3-OCH_3	H	20
5	4-OCH_3	H	343
6	H	H	16
7	2-Cl	H	33
8	2-CH_2CH_3	H	17
9	2-OCH_3	Cl	68



10

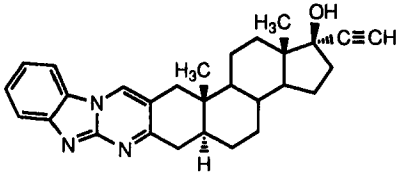
* IC_{50} value for displacement of [^{3}H]-SP from human IM-9 cells.

Subsequent to the initial studies reported for 2*S*,3*S*-**3**, a structurally novel nonpeptide SP antagonist RP-67580 (**17**) was disclosed (87). The SAR summary below highlights the increased receptor affinity afforded by the 2-methoxy group and amide to amidine modification (88). Compound **17** demonstrates potent *in vitro* (IC_{50} = 4.16 nM for NK_1 binding in rat brain) and *in vivo* activity (ED_{50} values for blockade of SP- induced plasma protein extravasation and phenylbenzoquinone writhing in mice of 0.04 mg/kg i.v. and 0.07 mg/kg s.c. respectively) (89). Other nonpeptide NK_1 receptor antagonists recently disclosed include the steroid-based **18** (NK_1 IC_{50} = 50 nM) (90), the glucose-based compound **19** (NK_1 IC_{50} = 60 nM) (91), and CGP-47,899 (**20**) (NK_1 IC_{50} = 10 nM) (92). Finally, the first nonpeptide NK_2 receptor antagonist SR 48,968 (**21**), with an IC_{50} value of 0.51 nM at the rat NK_2 receptor, was once again the result of an extensive medicinal chemistry effort around a lead uncovered by empirical screening (93). **21** blocks NKA dependent contraction of human isolated bronchus with a pA2 value of 9.4, and has no effect on contraction induced by histamine, acetylcholine, KCl, or $PGF_{2\alpha}$. This compound thus provides a potent and selective agent for exploring the role of NKA in pulmonary disease. Overall, these compounds provide an expanding opportunity to develop drugs which specifically target the NK_1 and NK_2 receptors.

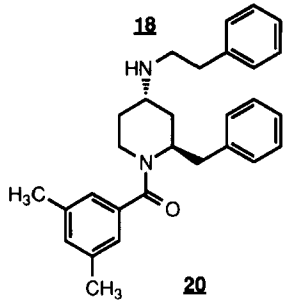


	Y	R	Z	X	SP IC_{50}*
11	H	H	O	H	181
12	OCH_3	H	O	H	75
13	H	CH_3	O	H	35
14	H	OH	O	H	113
15	H	H	O	4-CH_3	393
16	H	H	NH	H	36
17	OCH_3	H	NH	H	17

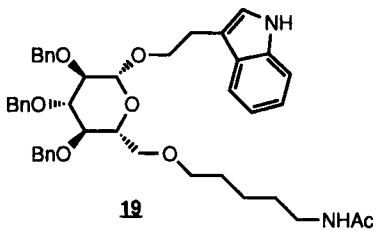
*IC_{50} value of displacement of [^{3}H]-SP from rat brain membranes



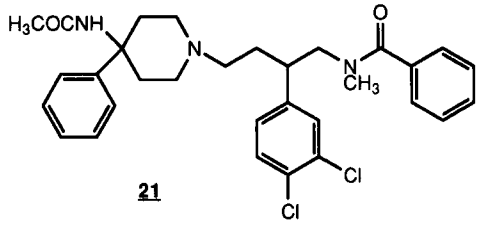
18



20



19



21

Tachykinin Antagonists in Pulmonary Disease — In early work, the peptidal antagonist spantide (94) showed inhibition of the bronchoconstriction and plasma extravasation elicited by vagal nerve stimulation, capsaicin or exogenous SP (95), as well as irritant-induced airway hyperreactivity (96). The availability of potent, selective antagonists has now set the stage for extensive assessment of the role of sensory neuropeptides in airway inflammation. 2*S*,3*S*-**3** inhibited airway plasma leakage induced by cigarette smoke (97); vagal nerve stimulation (98), and capsaicin or SP (99). Similarly, the bronchoconstriction elicited by exogenous SP in guinea pigs is selectively inhibited by 2*S*,3*S*-**3** (100,101). Importantly, however, the bronchospasm (increase in insufflation pressure) caused by capsaicin was blocked by NK_2 receptor antagonism and unaffected by 2*S*,3*S*-**3**, suggesting that both NK_1 and NK_2 mechanisms might participate in airway narrowing by different mechanisms (101). In a related study, 2*S*,3*S*-**3** had no effect on electrically-induced contractions of guinea pig hilar bronchus, while NK_2 antagonist peptides were effective (102).

The selective NK_2 antagonist **21** competitively inhibits the bronchoconstriction induced by NKA *in vivo* and *in vitro* (103,104). The combined NK_1/NK_2 antagonist **2** inhibited both the bronchoconstriction and plasma leakage in guinea pigs induced by capsaicin, SP or NKA without affecting the responses elicited by acetylcholine or histamine (105). Finally, in the first clinical trial to be reported in this field, **2** blocked the decrease in specific airway conductance induced by inhaled aerosolized bradykinin, which acts by causing release of tachykinins from C-fibers (106). These results suggest that the field is set to expand rapidly as the nonpeptide tachykinin antagonists are brought into the clinical arena.

Conclusions — Evidence for the involvement of tachykinin peptides in pulmonary disease is compelling. Both the tachykinin peptides and their receptors have extensive localization in pulmonary tissue, where they have been shown to mediate many of the characteristic aspects of pulmonary disease. The nonpeptide NK_1 and NK_2 antagonists are important new tools for assessing the potential therapeutic roles of tachykinin modulation in pulmonary diseases. The clinical trial with **2** already indicates the considerable potential in this field, which is poised for significant breakthroughs as more tachykinin antagonists are evaluated clinically.

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Chapter 12. Novel Approaches to Anti-Inflammatory Agents as Therapeutics for Pulmonary Disease

Michael J. Sofia and Steven A. Silbaugh
Lilly Research Laboratories
Indianapolis, IN 46285

Introduction - It has become well accepted that lung airway and tissue inflammation is a major aspect of the pathobiology of pulmonary diseases such as asthma, emphysema, adult respiratory distress syndrome (ARDS), cystic fibrosis and interstitial lung disease. For asthma, traditionally viewed as a brochoconstrictive disease, recognition of the importance of its inflammatory component is seen by the recent declaration by the National Institutes of Health that asthma should be treated foremost as an inflammatory disease (1). Evidence which supports a major role for an inflammatory component in pulmonary diseases such as asthma are the positive clinical effects of steroid therapy (2-4). However, steroid therapy suffers from documented side-effects, especially with chronic treatment (5,6). Consequently, development of novel entities which impact on the inflammation associated with pulmonary disease could constitute major therapeutic advancements.

Currently, new approaches directed toward treating the inflammation associated with pulmonary diseases have included the development of agents which impact on T-lymphocytes, mast cells, macrophages, neutrophils and eosinophils. By either their ability to produce other inflammatory mediators (e.g. cytokines, leukotrienes and cyclooxygenase products) which affect cell function or by their ability to release degradative enzymes, toxic proteins (e.g. eosinophil major basic protein, eosinophil cationic protein) and toxic oxygen metabolites, these cells have the capacity to participate in the inflammatory response (7,8). Novel approaches to anti-inflammatory therapy target the function of these cells by blocking cell adhesion, migration and activation. Other new agents inhibit the production or effect of inflammatory mediators, the release of toxic oxygen metabolites and proteins or inhibit degradative enzymes which some of these cells release. This chapter will deal with some major new approaches to anti-inflammatory therapy as they relate to pulmonary diseases.

REGULATION OF LIPID MEDIATORS

Lipid mediators constitute a major class of agents which modulate inflammatory cell function. Consequently, these agents continue to be the central focus of much research. Leukotriene B_4 (LTB_4) and platelet activating factor (PAF) are two products of arachidonic acid metabolism whose effects on inflammatory cells make them attractive targets for directing anti-inflammatory therapy. LTB_4 is a potent chemoattractant for both neutrophils and eosinophils (7,8). It activates neutrophils by inducing aggregation, calcium mobilization, superoxide release and up-regulation of CD11b/CD18 adhesion molecules (7-9). LTB_4 also causes the release of toxic oxygen metabolites by eosinophils (10). PAF activates neutrophils, eosinophils, lymphocytes and macrophages (11). It also promotes adhesion of various inflammatory cells to endothelium, and induces vascular leak and priming of these cells (11). This section will focus on inhibitors of phospholipase A_2 (PLA_2) enzymes, non-antioxidant and non-redox inhibitors of the 5-lipoxygenase enzyme (5-LO), inhibitors of 5-LO activating protein and antagonists of leukotriene B_4 (LTB_4) receptors, all agents which affect the production or antagonize the actions of LTB_4 or PAF. However, receptor-directed antagonists of PAF itself and leukotriene D_4 (LTD_4), which also has been implicated as a pro-inflammatory agent, will not be covered in detail. Numerous comprehensive reviews of both PAF and LTD_4 receptor antagonists have appeared recently (12,13).

Phospholipase A_2 Inhibitors - Phospholipase A_2 is the enzyme which catalyzes the production of arachidonic acid and lysophospholipids from phospholipids, and is the rate limiting enzyme in eicosanoid biosynthesis (14,15). PLA_2 is also known to be released from activated mast cells where it may participate in the degranulation process, and it is released from activated neutrophils and alveolar macrophages into interstitial and intravascular compartments (16,17). Elevated PLA_2

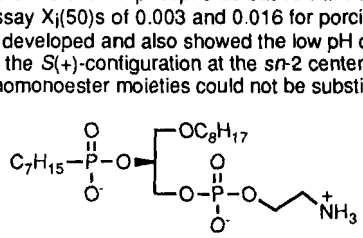
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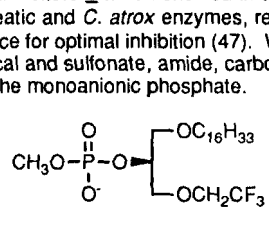
levels are documented in septic shock patients with concurrent ARDS (18). Increased levels are also found in brochoalveolar lavage (BAL) fluids of human asthmatics (19). Tracheal administration of PLA_2 to guinea pigs produces airway constriction and sequestration of neutrophils and eosinophils which have degranulated (20,21).

Phospholipases A have been isolated and purified from a number of sources, e.g. snake venoms, bee venom, porcine and bovine pancreas, rat inflammatory exudate and canine myocardium. To date, three human PLA_2 enzymes have been isolated, cloned and expressed *in vitro*. The human pancreatic group I PLA_2 is a 14 kDa enzyme (22). The human secreted PLA_2 ($sPLA_2$) is also a 14 kDa calcium-dependent protein of the group II type first isolated from human synovial fluid (23,24). Crystal structures of the human $sPLA_2$ have been determined to 2.2 Å resolution (24,25). This enzyme contains seven disulfide bonds and has a highly positively charged surface. The α-carbon positions and the functionally important and invariant residues His-48, Tyr-52, Tyr-73 and Asp-99 of the human $sPLA_2$ superimpose on that of the bovine pancreatic and *Crotalils atrox* enzymes, and the proposed nucleophilic water molecule, water-218, is H-bonded to the Nδ1 of His-48 (24). The human platelet enzyme is identical with the human $sPLA_2$ (26). The human cytosolic PLA_2 ($cPLA_2$) isolated from a U937 cell line is a 85 kDa protein with nanomolar calcium requirements (27-29). Assay of the PLA_2 enzymes is complicated by the need for phospholipases to act on aggregated substrates. Presently, three assay methods are used, the mixed micelle (MM) technique using either a radiolabelled or chromogenic substrate (30-33), the broken E. coli membrane (EC) method (34), and the anionic vesicle (AV) scooting mode method (35,36). The current trend in reporting enzyme inhibition data for PLA_2 enzymes is to report 50% inhibition as an $\alpha(50)$ = ([Inhibitor]/([Inhibitor]+[Substrate]) or $X_i(50)$ = [Inhibitor]/[Total Lipid] value. Methods also have been developed to assay the functional inhibitory effects of PLA_2 inhibitors on guinea pig tissues (37). It is obvious that due to the existance of multiple human PLA_2 isoforms, inhibitor selectivity has become an important issue for drug discovery.

Recently, reviews on inhibitors of phospholipases A have appeared (38,39). PLA_2 inhibitors can be divided into phospholipid-like and nonphospholipid-like structures. The phosphonate transition state inhibitor **1** was a potent inhibitor of both the human $sPLA_2$ (IC_{50} = 1.7 μM, EC) and pancreatic PLA_2 ($X_i(50)$ = 0.0013, AV) as well as the porcine pancreatic ($X_i(50)$ = 0.003, AV), the snake venom *Naja naja naja* ($X_i(50)$ = 0.0027, AV) and *A. halys blomhoffii* ($X_i(50)$ = 0.082, AV) enzymes (40,41). However, it was significantly less effective as an inhibitor of the U937 enzyme (41). Phosphonate **1** inhibited, in a concentration dependent manner, the contractile response induced by PLA_2 on guinea pig lung pleural strips (37) but failed to inhibit A23187 induced PGE_2 or LTC_4 production from human adherent monocytes or LTB_4 release from human neutrophils (41). X-ray structures of inhibitor **1** complexed with the *Naja naja atra*, bee venom, and human $sPLA_2$ enzymes confirmed **1** as a transition state analogue inhibitor (25, 42-44). Confirmatory solution evidence for the transition state mode of inhibition of **1** was provided by two-dimentional NMR studies (45) and enzyme studies which showed that a choline analogue of **1** had a higher affinity for the enzyme at low pH than at high pH, thus indicating the need for a bridging hydrogen between the oxygen of the phosphonate of **1** and the Nδ1 of His-48 in order to attain optimal affinity (46). An alternative class of *sn*-2 phosphorus-based transition state inhibitors **2** which showed in the anionic vesicle assay $X_i(50)$s of 0.003 and 0.016 for porcine pancreatic and *C. atrox* enzymes, respectively has been developed and also showed the low pH dependence for optimal inhibition (47). With these inhibitors, the *S*(+)-configuration at the *sn*-2 center was critical and sulfonate, amide, carboxy, oxime or phosphomonoester moieties could not be substituted for the monoanionic phosphate.



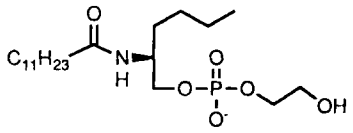
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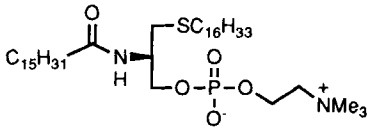
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Two *sn*-2 amide phospholipid transition state mimetics were demonstrated to be potent inhibitors of PLA_2 enzymes. Amide **3** showed a preference for mutant human platelet PLA_2 ($\alpha(50)$ = 0.0008, MM) over human pancreatic PLA_2 ($\alpha(50)$ = 0.01, MM) and showed a preference for phosphatidylglycerol over phosphatidylcholine or ethanolamine moieties at the *sn*-3 position (48, 49). The X-ray crystal structure of **3** complexed with a mutant porcine PLA_2 was determined, and

demonstrated that a hydrogen bond existed between the amide hydrogen of **3** and the Nδ1 of His-48, and that the amide carbonyl interacts with the acitve-site calcium (50). A series of *sn*-1 thioether *sn*-2 amides were studied as PLA_2 inibitors (51). Amide **4** was shown to bind analogously to **3** based on a 17-fold increase in affinity for PLA_2 at high pH which substantiates the formation of the critical H-bond with the active-site histidine (46).

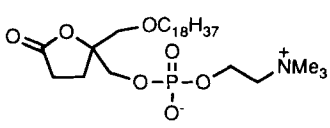


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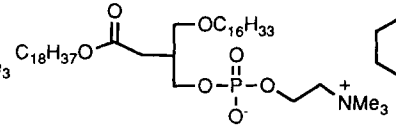


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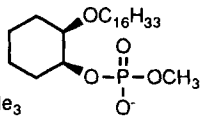
Attempts to design mechanism-based inhibitors of PLA_2 enzymes produced inhibitors **5** and **6** . These inhibitors, which were reported to show time-dependent inactivation, were designed, upon hydrolysis, to produce a bidentate ligand for calcium at the active site (52).



5



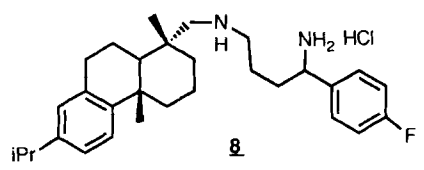
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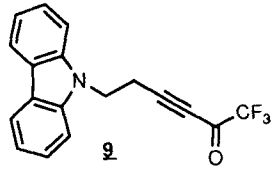
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An effort at designing a conformationally restricted phospholipid-like inhibitor, based on phosphate inhibitor **2**, resulted in the cyclohexyl phosphate ester **7**, which in the AV assay had $X_i(50)$s of 0.0005 and 0.0018 for porcine pancreatic and *C. atrox* PLA_2 enzymes, respectively (47). The *cis* orientation of the cyclohexyl substituents was optimal for potency, and it was found that the cyclohexyl could not be effectively replaced by phenyl (47).

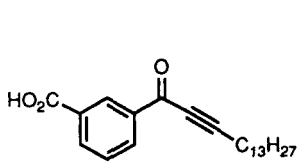
The non-phospholipid-like inhibitors of PLA_2 enzymes were derived from both synthetic and natural product sources. The synthetically derived entities are represented by inhibitors **8** to **12**. In a sonicated phospholipid assay using either the porcine pancreatic or rat polymorphonuclear PLA_2, **8** inhibited with IC_{50}s of 1.8 and 1.9 μM, respectively (53). The derivative with a hydroxyl group in place of the primary amine was 10-fold less active (53). Inhibitor **8** was also shown to be active in the rat carrageenan paw edema model of inflammation with an oral ED_{50} of 4.8 mg/kg (53). Structure **9**, the most active compound in a series of alkynyl fluoroketones, inhibited the human $sPLA_2$ (IC_{50} = 0.043 μM, EC) and was also active *in vivo* in a PLA_2 murine paw edema assay (ED_{50} = 1 mg/kg, ip) where PLA_2 was administered exogenously (54). Alkynyl ketones, in which structure **10** is a representative, also inhibited human $sPLA_2$ (IC_{50} = 11 μM, EC) and inhibited the synthesis of LTB_4 in HL60 cells (55). The central phenyl moiety in **10** was effectively replaced with a 2,6-substituted pyridine or 2,5-substituted thiophene unit (56). The indole 3-acetic acid **11** (WAY-121,520), which was derived from a series of indole-based 5-LO inhibitors, inhibited the human $sPLA_2$ in the *E. coli* membrane assay with an IC_{50} of 4 μM (57). When assayed using a non-aggregated phospholipid substrate, benzenesulfonamide **12** inhibited a membrane preparation of a rat heart PLA_2 with an IC_{30} of 0.009 μM (58). *In vivo*, benzenesulfonamide **12** was reported to inhibit ischemic myocardial injury in rats via blockade of PLA_2 (58). Various fatty acid amides were reported as potent and selective inhibitors of various PLA_2 enzymes (59).



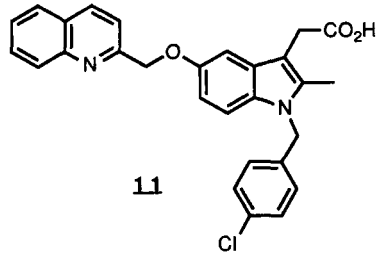
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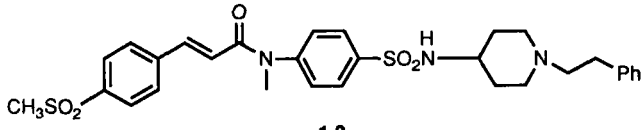
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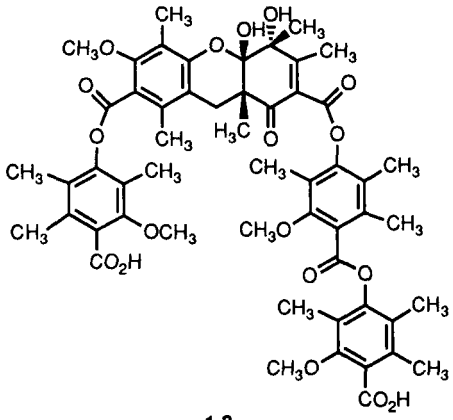


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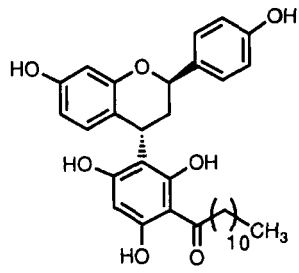


12

Two natural products were disclosed as PLA_2 inhibitors, thielocin A1ß and YM-26567-1 (13 and 14). Thielocin A1ß isolated from the fermentation broth of *Thielavia terricola* RF-143 showed pronounced selectivity for the rat platelet enzyme (IC_{50} = 0.0033 µM, MM) over the human $sPLA_2$, human pancreatic, several snake venom and the bee venom enzymes (60). It was also reported to be a noncompetitive inhibitor of the rat enzyme (60). Compound 14 obtained from the fruit of *Horsfieldia amygdaline* was 8-times more potent as a competitive inhibitor of the rabbit platelet enzyme (Ki = 1.6 µM, EC) than as an inhibitor of the porcine pancreatic enzyme (61). *In vivo* in a model of TPA induced ear edema, 14 caused a dose dependent reduction in swelling with an ED_{50} = 28 µg/ear (61).

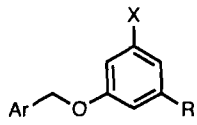


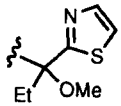
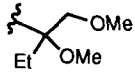
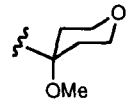
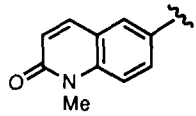
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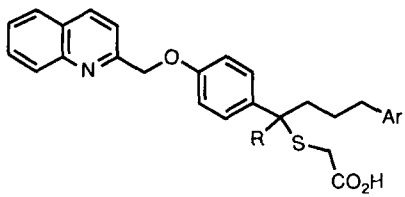
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5-Lipoxygenase (5-LO) Inhibitors - Pharmacological intervention at the conversion of arachidonic acid to leukotriene A_4 by the development of 5-LO inhibitors is a clinically viable approach for anti-inflammatory therapy and has shown efficacy in clinical asthma (62,63). Reviews on 5-LO inhibitors have appeared (64,65). Inhibition of 5-LO activity can occur either by direct enzyme inhibition or inhibition of the 5-lipoxygenase activating protein (FLAP). A series of non-redox (methoxy-alkyl)thiazoles 15 (ICI-211965) showed enantiospecific interaction with the enzyme but demonstrated limited oral activity. Based on structure 15 and via dimethyl glycol ether 16, methoxytetrahydropyran 17 (ICI D2138) was developed (66,67). Optimization of the *ex vivo* potency of this series came by modification of the aryl substituent from 2-naphthyl (ED_{50} = 10 mg/kg at 3h) to the *N*-methyl-2-quinolone (ED_{50} = 0.9 mg/kg at 3h) moiety (66). When administered iv, 17 (ID_{50} = 0.1 mg/kg) showed a dose-dependent inhibition of antigen-induced bronchoconstriction in guinea pigs (68).

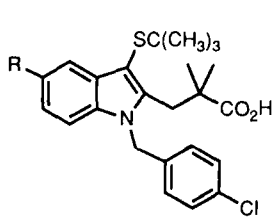


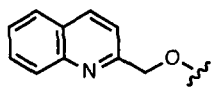
	R	X	Ar	Human Whole Blood IC50 (μM)
15		H	2-Naphthyl	0.4
16		H	2-Naphthyl	0.4
17		F		0.02

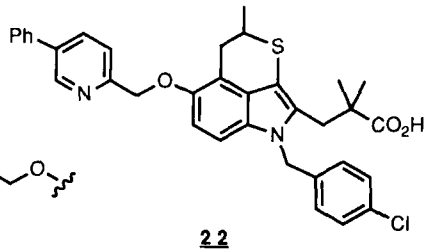
The demonstration that for the FLAP inhibitor **18** (L-674,636), only a two-fold differential existed for the inhibition of leukotriene synthesis by the (-)- and (+)-enantiomers (IC_{50}s = 5 and 10 nM , respectively), and that analogue **19** was intrinsically more potent, resulted in the postulation of a receptor model for FLAP (69). This model proposed two lipophilic regions adjacent to an acid binding site (69). Correlation of this model with the indole based FLAP inhibitor structure **20** (MK-886) produced the quinolinylmethoxy derivative, inhibitor **21** (L-686,708; MK-0591). Compound **21** was more effective than **20** at both inhibiting leukotriene synthesis in human whole blood and binding to FLAP protein (70). When evaluated in a sheep asthma model, **21** inhibited both the early and late-phase bronchoconstriction (71). This inhibition correlated with the reduction in urinary leukotriene E_4 excretion (71).



	R	Ar
18	H	4-Cl-Ph
19	$(CH_2)_2Ph$	Ph



	R
20	$(CH_3)_2CH$
21	

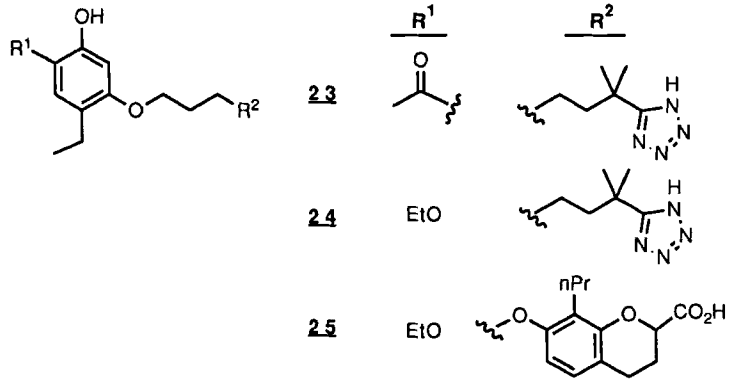


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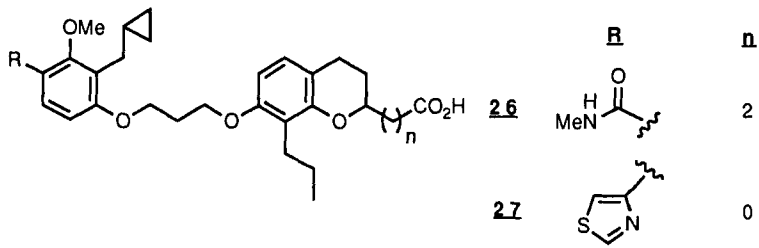
Studies showed that either substitution of the quinoline of **21** with a phenylpyridyl unit or replacement of the indole with a thiopyranoindole core structure produced direct 5-LO enzyme inhibitory activity (72). Combination of these observations produced a selective 5-LO enzyme inhibitor **22** (IC_{50} = 60 nM, L-689,065) which showed a 3.5-fold preference for the (-)-enantiomer and was orally active in a rat model of hyperreactive airways (72).

Leukotriene B_4 Receptor Antagonists - A review on LTB_4 antagonists has appeared (73). Recently, development of LTB_4 receptor antagonists as general anti-inflammatory agents has received significant attention. Although early work focused on LTB_4 mimetics, current efforts primarily target non-mimetic structures. The 1,2,4,5-substituted hydroxyacetophenone **23** (LY255283) antagonized LTB_4-induced functional responses *in vitro* in both human neutrophils and guinea pig eosinophils (9,

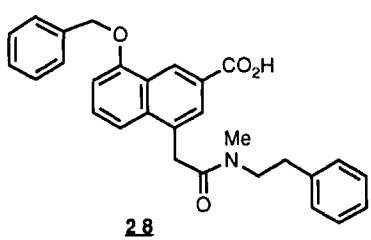
74-76). It was also demonstrated to inhibit, via oral administration in the guinea pig, LTB_4-induced bronchoconstriction and antigen-induced eosinophilia (75,76). The *ortho*-ethoxy derivative **24** (LY247833) produced a 17-fold improvement in intrinsic receptor binding (IC_{50} = 4.8 nM) and a 10-fold improvement in *in vivo* efficacy (77). Antagonist **25** (LY282201) showed that substitution of other structurally different lipophilic acids for the *gem*-dimethyl tetrazole acid unit provided enhanced receptor binding and increased *in vivo* potency using a model of LTB_4-induced airway obstruction (78).



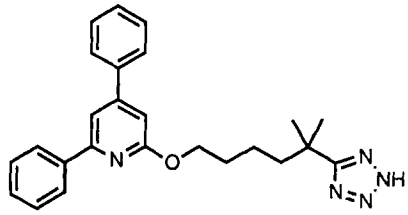
Efforts to optimize the potency of the 1,2,3,4-substituted methylated phenol class of antagonists, originally exemplified by the methylated hydroxyacetophenone SC-41930, focused on the two *ortho*-methoxy substituents. The amide derivative **26** (SC-51146) showed at least a 10-fold improvement in both receptor binding and antagonism of chemotaxis (73,79). Heterocyclic derivatives, exemplified by **27** (SC-50605), also provided improvements in receptor binding and antagonism of chemotaxis with low nanomolar IC_{50}s (73,80). *In vivo*, **27** was effective at inhibiting intradermal granulocyte trafficking (80).



Other mono-acid antagonists are the trisubstituted naphthoic acid **28** (RG-14893) and the trisubstituted pyridine **29** (RP69698). Naphthoic acid **28** had a Ki of 2 nM for binding to human neutrophils, and in the monkey, it inhibited neutropenia and neutrophilia 61% and 73%, respectively at a 3 mg/kg iv dose (81) . Antagonist **29** inhibited LTB_4 binding to human neutrophils with an IC_{50} of 20 nM (82).

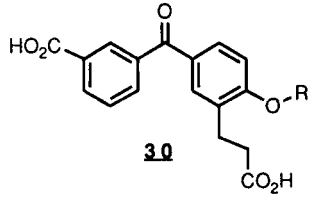


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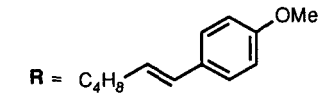


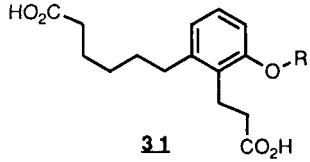
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Diacids **30** (LY223982), **31** (ONO-4057), and **32** (LY264086) are grouped as an entirely separate class of LTB_4 receptor antagonists due to the fact that both acids are critical for potency (83,84,85). The second acid may bind to a site at the receptor which is unrelated to the binding of LTB_4 itself or to the binding of any other class of antagonist. Investigation of the preferred orientation of the two acid substituents was accomplished by use of a conformationally rigid xanthone template (85). The preferred orientation is that shown by the xanthone analogue **32**.

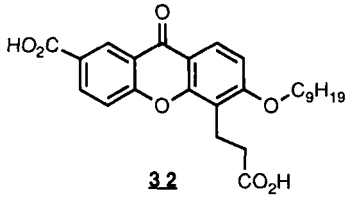


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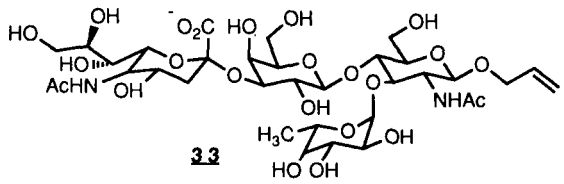


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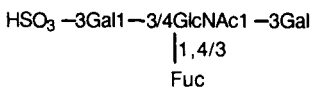
ADHESION MOLECULES

Before leukocytes can transit from the blood to pulmonary tissue, they must first adhere to the vascular endothelium. Close cell-to-cell interactions are critically important in this aspect of the inflammatory process. These intercellular communications are mediated by specific cell-surface glycoproteins, termed adhesion molecules, which are up-regulated in response to agents such as cytokines and leukotrienes (86). For example, leukocytes are first slowed by a selectin-mediated process and roll along the endothelial cell wall (86, 87). A firmer attachment results when the neutrophil integrin LFA-1 binds to ICAM-1 or other adhesion molecules located on the endothelial surface. Other molecules, such as MAC-1 (CD11b/CD18), may be important for the binding and emigration of neutrophils from the vascular bed to the inflammation site (88). A monoclonal antibody to ICAM-1 (MAb 6.5) reduces antigen-induced airway eosinophilia and airway hyperresponsiveness in a primate asthma model (89). An antibody (MAb CL2) to the endothelial cell selectin ELAM-1 (E-selectin) inhibited neutrophil infiltration and late onset airway obstruction in the same test system (88). A monoclonal antibody to CD18 (R15.7) has also been reported to inhibit leukocyte infiltration and increased airway responsiveness in the guinea pig (90).

One carbohydrate adhesion molecule of intense interest is sialyl Lewis^x (**33**), a tetrasaccharide terminus of some gylcoproteins and glycolipids (91, 92). Sialyl Lewis^x, which is located on leukocyte surfaces, can bind to adhesion molecules such as E-selectin expressed on the endothelial cell surface. Potential therapeutic approaches include using sialyl Lewis^x itself or simplified structures based on sialyl Lewis^x to bind to endothelial cell receptors and thereby prevent leukocyte adhesion. Another potential approach now under investigation is the use of nucleoside aza-sugars to block fucosyltransferase, an enzyme needed for sialyl Lewis^x biosynthesis (93). A sulfate-containing oligosaccharide ligand **34** for the human E-selectin molecule has also been described (94).



33



34

CYTOKINE INHIBITION AND ANTAGONISM

Cytokines are intercellular regulatory proteins that differ from classical hormones in that they are usually made by more than one cell, have a wide range of biologic effects and generally act over short distances (95). It is possible that overproduction of specific cytokines contributes to the pathogenesis of pulmonary inflammatory diseases. Although it is not yet clear which cytokines are important in pulmonary disease, it seems likely that agents which influence the synthesis or action of specific cytokines would be useful in treating lung inflammation. The therapeutic potential of cytokine modulation is supported by recent studies in animals and man (96-98).

A naturally occurring interleukin 1 receptor antagonist (IL-1ra) has been cloned and the recombinant protein appears to compete with IL-1 for cell-surface receptors (99). IL-1ra partially inhibited neutrophil accumulation and airway hyperresponsiveness following antigen challenge in the guinea pig (96). Treatment of guinea pigs with purified rat anti-mouse IL-5 reduced the antigen-induced influx of eosinophils into the lung (97).

Another potential therapeutic approach is inhibition of cytokine synthesis. Drugs such as cyclosporin A and FK506 may reduce pulmonary inflammation by virtue of their ability to suppress cytokine generation from T-lymphocytes and mast cells (100). Both agents reduce antigen-induced lung eosinophilia in the guinea pig (101). A recent clinical trial which examined the effects of cyclosporin A in steroid-dependent asthmatics was particularily encouraging. Patients treated with cyclosporin exhibited improved lung function and a reduced frequency of disease exacerbations (98, 102). Rapamycin is structurally similar to FK506 and also inhibits antigen-induced eosinophil accumulation in the guinea pig (103). Other agents have been reported to inhibit interleukin synthesis or release and have recently been reviewed (104).

Conclusion - Although much remains to be learned concerning the pathobiology of the inflammatory process, therapeutic approaches related to the regulation of lipid mediators, adhesion molecules and cytokines are clearly justified based upon our current knowledge of pulmonary disease. It is not possible to predict which specific approaches will yield useful drugs. However, it seems likely that at least some of the medicinal chemistry strategies summarized in this chapter will ultimately prove to be clinically viable.

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SECTION III. CHEMOTHERAPEUTIC AGENTS

Editor: Jacob J. Plattner
Abbott Laboratories, Abbott Park, IL 60064

Chapter 13. Recent Advances in Anti-Infective Agents

Milton L. Hammond
Merck Research Laboratories
P.O. Box 2000
Rahway, NJ 07065

Introduction - Over the last year the specter of increased resistance to established antibiotics has continued to provide a major incentive for the development of new antimicrobial agents (1-3). In addition, the increasing incidence of opportunistic infections in immunocompromised individuals has created a need for effective therapy for these heretofore unusual pathogens. This chapter will review the major developments and new agents reported in the antibacterial and antifungal literature within the last year. The biochemical mechanisms of antimicrobial resistance are the topic of Chapter 17 in this Volume (4).

ANTIBACTERIAL AGENTS

β-Lactams - A review of the antibacterial properties and clinical usage of imipenem (1) / cilistatin has appeared (5) as has a comparison of the *in vitro* and *in vivo* antibacterial activities of meropenem (SM 7338, 2) with 1 and a variety of cephalosporins (6). An *in vitro* comparison of LJC 10,627 (biapenem, 3) with 1 and 2 showed 3 to have generally similar activity to 1 (7). In three pharmacokinetic studies, 2 showed an increased terminal half life in the elderly (8) and patients with renal impairment (9, 10) when compared with appropriate control groups. A comparison of the sensitivity of 1 and 2 to renal dehydropeptidase-1 (DHP-1) obtained from a variety of species revealed that the relative sensitivities varied greatly (11). However, when the effect of 1-β-methyl substitution was examined by comparing 1 with its 1-β-methyl analogue (4), and 2 with the corresponding desmethyl compound (5), it was found that the introduction of the 1-β-methyl substituent reduced the DHP-I sensitivity against both the mouse and swine enzymes primarily by reducing Vmax. Similar results were obtained when 3 was compared to its desmethyl analogue 6 (12). The penem analogue of 2 (7) showed an increased sensitivity to DHP-I relative to 2 (11).

Synthesis of a series of carbapenems with dibasic side chains at the 2-position resulted in the discovery of the broad spectrum agent DX 8739 (8) which was 2 to 4-fold more potent than 2 against 51 clinical isolates of *Pseudomonas aeruginosa* (13). In murine *P. aeruginosa* models, 8 was superior to 2 both against a systemic infection and in a pneumonia model induced via an aerosolized challenge (14). Pharmacokinetic evaluation of 8 in monkeys revealed a half-life of 29.9 min and a mean urinary recovery of 3.8%, both of which were improved to 40.7 min and 83.6%, respectively, by coadministration of cilistatin (15).

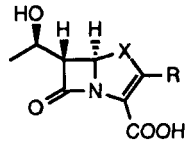
The exploration of a series of tricyclic β-lactams, tribactams, resulted in the selection of GV 104326 (9) as a development candidate (16). *In vitro* 9 exhibited an antibacterial spectrum similar to 1, except for *Pseudomonas* (17), and had increased stability to human DHP-I when compared with 1 or 2 (18). The hexetil ester of 9 (GV 118819, 10) exhibited excellent oral bioavailabilitiy in mice and rats which resulted in superior oral efficacies (ED_{50}s <1.3 mg/kg) in mouse protection tests against gram-positive and gram-negative challenges (19).

Examination of a series of 1-substituted 2-methylthio-2-[1,3,4]-thiadiazole carbapenems (11-14) revealed that 1-fluoromethyl and cyanomethyl substituents were superior to 1-hydroxymethyl or 1-acetoxymethyl substituents but had no advantage over the corresponding 1-methylcarbapenem. In all cases the 1-β orientation was superior to the corresponding 1-α analogue (20). The synthesis of

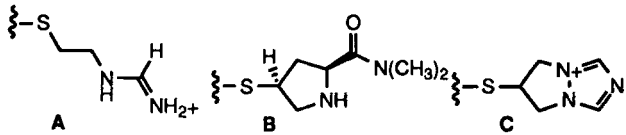
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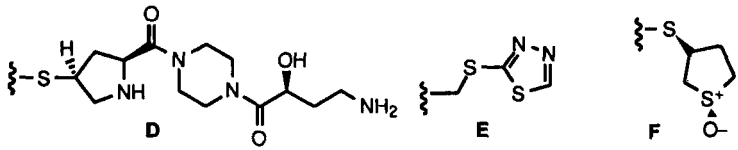
a series of 6-substituted 1-β-methyl-2-methylthiopyridine carbapenems revealed that the 6-hydroxymethyl (15) and 6-aminomethyl (16) analogues had superior *in vitro* antimicrobial activity to *their 6-(1'R-hydroxyethyl) (17) and 6-(1'R-aminoethyl) homologues (18). The 6-aminosubstituted* carbapenems 16 and 18 had lower gram-positive activity, similar gram-negative activity, but increased activity against *Pseudomonas* species relative to the 6-hydroxy substituted analogues 15 and 17 (21).



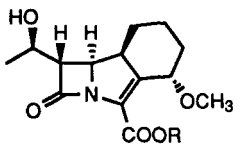
	X	R		X	R
1	CH_2	A	8	β-$CHCH_3$	D
2	β-$CHCH_3$	B	11	β-$CHCH_3$	E
3	β-$CHCH_3$	C	12	β-$CHCH_2F$	E
4	β-$CHCH_3$	A	13	β-$CHCH_2CN$	E
5	CH_2	B	14	β-$CHCH_2OH$	E
6	CH_2	C	21	S	F
7	S	B			



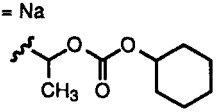
A B C

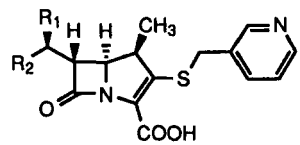


D E F

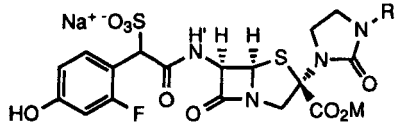


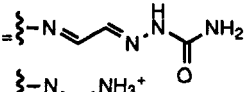
9 R = Na

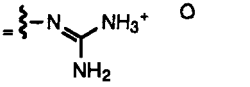
10 R= 



15 R_1 = OH R_2 = H
16 R_1 = NH_2 R_2 = H
17 R_1 = OH R_2 = CH_3
18 R_1 = NH_2 R_2 = CH_3



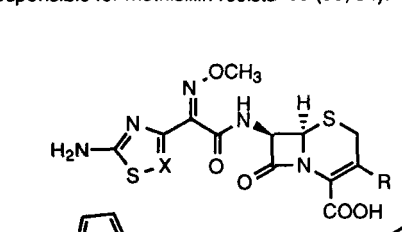
19 M = Na + R = 

20 M = – R = 

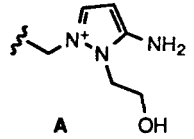
Evaluation of a large number of 2-carboxypenams with a variety of substituents at the C-2 and C-6 positions resulted in the selection of T 5575 (19) and T 5578 (20) as narrow spectrum antibiotics with potent activity against gram-negative species (22). *In vitro* 19 exhibited activity superior to ceftazidime and aztreonam against panels of *E. coli, K. pneumoniae, E. cloacae*, and *P. aeruginosa*. In contrast, 20 was 50 to 300-fold less potent than 19 except against *P. aeruginosa* where 20 was slightly superior to 19. Both compounds had activity against ceftazidime and imipenem-resistant strains and exhibited high affinity for the penicillin binding protein 3 (PBP-3) of *E. coli* and *P. aeruginosa* (23). In the penem area an efficient synthesis of sulopenem (CP 70,429, 21) has been described (24). *In vitro* 21 is slightly less active than imipenem against susceptible gram-positive

organisms but four to eight-fold more potent against gram-negative bacteria (25), except *P. aeruginosa* (26, 27).

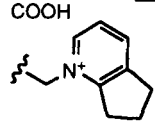
Recently several new parenteral cephalosporins have appeared. FK 037 (**22**) has a broad antibacterial spectrum, similar to cefpirome (**23**) but with greater activity against methicillin-resistant strains of *S. aureus* (MRSA) (28). Against a broad panel of MRSA strains **22** exhibited an MIC_{90} of 16 μg/mL (29, 30), inferior to vancomycin (MIC_{90} = 2 μg/mL). Nevertheless, **22** was able to significantly reduce colony forming units (CFUs) in an MRSA soft tissue infection model (30) and exhibited superior activity to imipenem and **23** against a lethal systemic infection of MRSA (31). Synthesis of a series of 3-(benzothiazol-2-yl)-thiocephalosporins resulted in the discovery of CP 0467 (**24**) with *in vitro* MRSA activity (MIC_{90} = 6.25 μg/mL) greater than ten-fold superior to **1** and about one quarter that of vancomycin (MIC_{90} = 1.56 μg/mL) (32). Consistent with its unusual MRSA activity, **24** had a high affinity for PBP-2' (IC_{50} = 0.49 μg/mL) from *S. aureus* (32), the expression of which is responsible for methicillin resistance (33, 34).



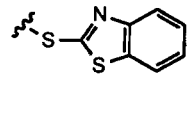
	X	R
22	CH	A
23	CH	B
24	N	C
25	N	D
26	N	E
27	N	F
28	CH	D



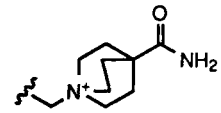
A



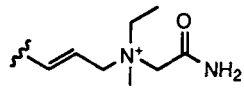
B



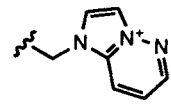
C



D

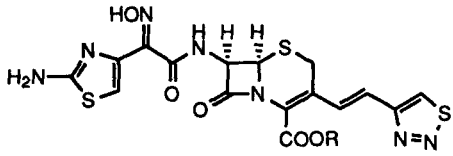


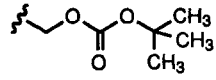
E



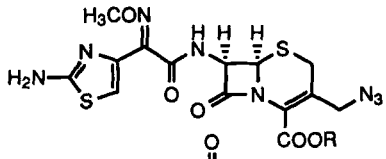
F

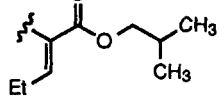
Several of the newer parenteral cephalosporins, such as ceclidin (E 1040, **25**), E 1077 (**26**), and SCE 2787 (**27**), exhibit improved activity against *P. aeruginosa* and possess a 7β-(α-oxyiminoaminothiadiazolyl) group which replaces the more traditional 7β-(α-oxyiminoaminothiazolyl) group. A comparison study of **25** with **28**, in which the aminothiadiazole was replaced with an aminothiazole, concluded that the increased anti-pseudomonal activity stems primarily from a decreased affinity for the chromosomal cephalosporinase. In addition, the more hydrophilic aminothiadiazole may lead to increased penetration of the outer membrane proteins in *P. aeruginosa* (35). Several additional reports dealt with the *in vitro* and *in vivo* antibacterial activities of **25**, **26**, and **27**. Among the β-lactams tested against an extended panel of imipenem-resistant *P. aeruginosa* strains, **25** exhibited the most potent activity (36). In a model of refractory *P. aeruginosa* induced urinary tract infection in rats, **25** was markedly superior to ceftazidime in eradicating the infecting organism from the urine (37). Three reports compare the antibacterial spectrum of **26** (38-40) with cefpirome (**23**) and other β-lactams. In general, **26** exhibits similar activity to **28** but is two to four-fold more potent against *E. faecalis*, *E. cloacae*, and *P. aeruginosa*, while **28** is more potent against *Proteus vulgaris*. The preparation and antibacterial evaluation of a series of 3-(condensed heterocyclic azolium) methyl cephalosporins leading to **27** revealed a similar result to that described above; the 7β-(α-oxyiminoaminothiadiazolyl) group generally resulted in improved activity against *P. aeruginosa* compared to the corresponding aminothiazole or 5-chloro-2-aminothiazole (41), which was the optimal thiazole substituent (42). The antibacterial properties of **27** were similar to cefpirome (**23**) except against *P. aeruginosa* where **27** was somewhat more potent *in vitro* and *in vivo* (43-45). In comparative pharmacokinetic studies, the half-life of **27** varied from 0.2 to 0.3 hrs in mice and rats to 1.3 hrs in cynomolgus monkeys and was excreted mainly unchanged in the urine (46). A systematic structure-activity study of a series of cephalosporins containing C-3' catechol *ton*-B pharmacophores showed that increasing the acidity of the catechol led to both improved β-lactamase stability and superior pharmacokinetic behavior in marmosets (47, 48).



29 R = 

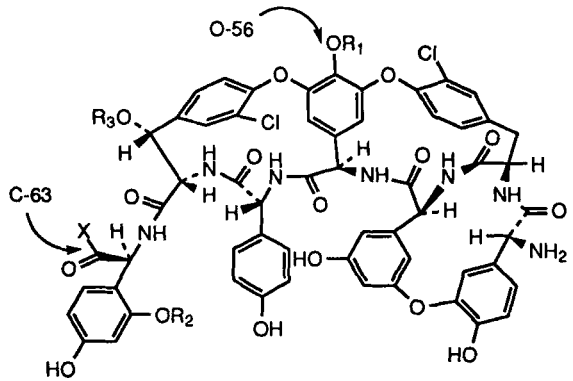
30 R = H



31 R = 

32 R = H

Several new oral cephalosporins have been described including SC 004 (**29**), the pivaloyloxymethyl (POM) ester of SC 002 (**30**), and Ro 41-3199 (**31**). *In vitro* **30** was comparable to cefdinir (49) while **29** compared favorably to cefdinir in septicemia models after oral administration (50). A comparison of the oral bioavailabilities of a number of prodrug esters of Ro 40-6890 (**32**) in rats and mice led to the selection of the novel isobutoxycarbonylpentenyl ester, **31**, as a development candidate (51). *In vitro* **32** was slightly superior to cefpodoxime against a variety of gram-positive and gram-negative species, but was slightly inferior to the parenteral agent ceftriaxone (52). In murine septicemia models the *in vivo* results with **31** generally paralleled the *in vitro* results, with **31** being equal or superior to cefpodoxime proxetil, particularly against gram-negative pathogens (52). When the *in vitro* activity of **32** was evaluated against 164 clinical isolates of predominantly intestinal bacteria of the families *Enterobacteriaceae* and *Vibrionaceae*, **32** was found to be similar to cefotaxime and superior to amoxicillin / clavulinic acid (53). In a Phase I study in healthy male volunteers, the half-life of **32** was 1.9 hours and the bioavailability of **31** varied from 19-34% depending on the formulation (54).



33 R_1 = N-acyl-β-D-glucosaminyl NHCOR side chain

34 R = $(H_2C)_2$-C(H)=C(H)-$(CH_2)_4CH_3$ **37** R = $(CH_2)_6CH(CH_3)CH_2CH_3$

35 R = $(CH_2)_6CH(CH_3)_2$ **38** R = $(CH2)_7CH(CH_3)_2$

36 R= n-C_9H_{19}

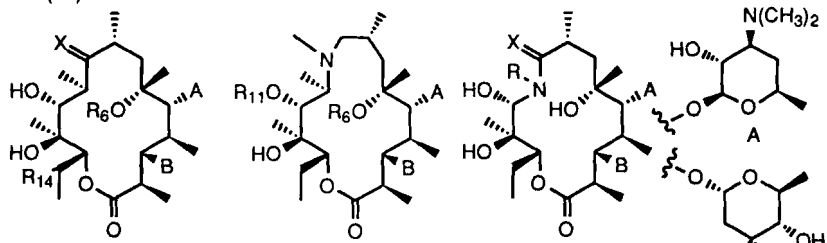
R_2 = α-D-mannosyl; R_3 = N-acetyl-β-D-glucosaminyl; X = OH

39 $R_1 = R_2 = R_3$ = H X = $NH(CH_2)_3N(CH_3)_2$
40 R_1, R_2,and R_3 as in **33** X = $NH(CH_2)_3N(CH_3)_2$
41 R_1, R_2,and R_3 as in **35** X = $NH(CH_2)_3N(CH_3)_2$
42 $R_1 = R_2 = R_3$ = H X = OH

Glycopeptides - Efforts continue in the glycopeptide area concentrating on the goals of improving activity against less sensitive coagulase negative staphylococci and enterococci and broadening the antibacterial spectrum to include gram-negative pathogens. Teicoplanin (**33**) is a mixture of five

major components, A2-1 (34), A2-2 (35), A2-3 (36), A2-4 (37), and A2-5 (38) (55). A study of the metabolism of 33 in humans has identified two minor metabolites as arising from the oxidation of 36 at the 8 and 9 positions of the decanoic acid chain (56). The *in vitro* evaluation of three previously reported (57) C_{63} amide derivatives of 33, MDL 62208 (39), MDL 62211 (40), and MDL 62873 (41), against a large panel of gram-positive organisms revealed that these compounds were superior to 33 or vancomycin and had good activity against some teicoplanin-resistant coagulase negative staphylococci, but were ineffective against vancomycin-resistant enterococci (58). A QSAR analysis of a series mono and polyamine C_{63} carboxamides of 33 and its aglycone (42) has emphasized the importance of lipophilicity and increased basicity in optimizing *in vivo* activity in a *Strep. pyogenes* septicemia model (59). Evaluation of a series of basic amides of 33 or 42 resulted in the discovery of unexpected gram-negative activity (60). Synthesis of a series of O_{56} carboxyesters and carboxyamides of 42 showed no improvement in activity when compared to 42 (61).

Macrolides - Several reviews have appeared describing the antimicrobial properties of clarithromycin (43) and azithromycin (44) (62-65). The absolute bioavailability of 43 in man was 55% (66), but taking into account the considerable first pass metabolism of 43 to the 14-hydroxymetabolite 45 (67) led to the conclusion that 43 was almost completely orally absorbed. The pharmacokinetics of 43 in man was dose dependent with a terminal half-life ranging from 2.3 to 6.0 hrs over a dose range of 100 to 1200 mg (67). The presence of 40% human serum was found to potentiate the antibacterial activity of 44 26-fold and 15-fold for serum-resistant *E. coli* and *S. aureus*, respectively. A lesser potentiation was observed for erythromycin (46) while the presence of human serum antagonized the activity of the more highly protein bound macrolide, roxithromycin (47) (68). Within one hour neutrophils and blood leukocytes accumulated 160-fold the extracelluilar concentration of 44 *in vitro* while *in vivo* intracellular concentrations of 44 reached 45.2 µg/mL two hours after a third dose and declined to 36.6 µg/mL a week later. The corresponding serum concentrations were 0.2 and <0.05 µg/mL, respectively (69). The uptake and transport of 44 by phagocytes has been reviewed (70).



43 $R_6 = CH_3$ $R_{14} = H$ X = O
44 $R_6 = H$ $R_{11} = H$
48 R = CH_3 X = H, H
45 $R_6 = CH_3$ $R_{14} = OH$ X = O
51 $R_6 = H$ $R_{11} = CH_3$
49 R = H X = H, H
46 $R_6 = H$ $R_{14} = H$ X = O
52 $R_6 = CH_3$ $R_{11} = H$
50 R = H X = O
47 $R_6 = H$ $R_{14} = H$
X = $NOCH_2O(CH_2)_2OCH_3$

Substantial effort in the past year has been directed at defining the therapeutic spectrum of the newer macrolides, particularly against such opportunistic infections as *Mycobacterium avium* and *Toxoplasma gondii*. *In vitro* the MICs of 43 were less than 4 µg/mL, the maximum plasma concentration *in vivo*, against 14 of 18 species of *Mycobacteria* at pH 7.4 (71); 43 was 10 to 50 times more active than 46 against the rapidly growing mycobacterium *M. chelonae* (72). The inhibitory and bactericidal concentrations of 43 against intracellular populations of *M. avium* in human macrophages were similar to results obtained by broth dilution at pH 7.4 (73), and a combination of 43, its metabolite 45, and minocycline was more effective than 43 alone (74). Similarly, 43 was more effective than 46 against intracellular populations of *M. avium* in murine macrophage derived J774 cells and in alveolar macrophages isolated from HIV-1 infected individuals. Combinations of 43 with ethambutol and rifampin showed greater activity than 43 alone (75). In an *in vivo M. avium* infection model in beige mice, both 43 (76) and 44 (77) were similarly effective at doses of 50 to 200 mg/kg/day, and the activity of both compounds was enhanced in combination with rifabutin. However, monotherapy with 43 in the beige mouse resulted in the selection of resistant mutants (78). The results of several clinical trials of 43 for the treatment of disseminated *M. avium* infection have been promising, but the development of resistance, as measured by increases in the MIC, has been observed (79-82).

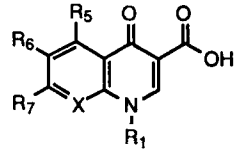
In a murine model of *Toxoplasma gondii* infection **44** prolonged survival of infected outbred Swiss mice at doses of 75 to 300 mg/kg/day po. Examination of the tissues from these animals showed that **44** rapidly reduced the parasite burden from the blood and lungs at all doses but was unable under these conditions to reduce the parasite burden in the brain. Administration of **44** (150 mg/kg/day) in combination with sulfadiazine or pyrimethamine, drugs whose effectiveness is known to be limited to the period of therapy, resulted in a remarkable synergism which was quite effective at removing the cyst burden from the brain and preventing relapses after the cessation of therapy (83). Qualitatively similar results have been obtained in other studies with **44** (84) and **43** (85). A combination of **43** and the tetracycline, minocycline, substantially reduced the parasite burden in the brain in a murine toxoplasmosis model. Since this combination does not contain a folate inhibitor, it may have a decreased incidence of side effects (86). As a prelude to the use of **44** prophylactically, a recent Phase I trial in HIV-infected patients showed that **44** (1 g once a week for 5 weeks) did not alter the distribution of zidovudine, and exhibited similar pharmacokinetics after repeated administration (87).

The preparation of the 14-membered azalide **48** (88) has allowed a direct comparison of the *in vitro* antimicrobial activities of **48** and its 15-membered homologue **44** (89). While **48** exhibited a similar antimicrobial spectrum to **44**, it was slightly less active. Analogues of **48** exhibited a similar structure-activity pattern to that previously described for **44** (90) with the unmethylated azalide **49** being slightly less active, and the corresponding lactam **50** being devoid of antibacterial activity. The preparation and evaluation of a series of methylated analogues of **44** revealed that the 11-O-methyl analogue **51** was up to four-fold more potent than **44**, while the 6-O-methyl analogue **52** was generally slightly less potent than **44** (91).

Streptogramins - Pristinamycin is a naturally occurring mixture of pristinamycin Ia (**53**) and pristinamycin IIa (**54**) which has broad activity against gram-positive pathogens. Pristinamycin has been available in Europe for over 25 years but its use has been restricted due to its very low water solubility. An effort to provide water soluble analogues of pristinamycin resulted in the development of RP 59500 (**55**), a 30:70 mixture of the semisynthetic analogues RP 57669 (**56**) and RP 54476 (**57**) (92). As with **53** and **54**, **56** and **57** individually possess weak bacteriostatic activity, but in combination exhibit pronounced synergy resulting in potent bactericidal activity. This synergistic effect is likely due to the formation of a stable 1:1:1 ternary complex of **56**, **57**, and the 50S subunit of the bacterial ribosome. It has been proposed that **57** binds to the L_{10} and L_{11} proteins of the bacterial ribosome which induces a conformational change that increases the affinity of **56** for the L_{24} subunit of the ribosome (93). *In vitro* **55** has good activity against a wide variety of gram-positive organisms, including macrolide and methicillin resistant strains (94). Against enterococci **55** was effective against vancomycin sensitive strains of *E. faecium* (MIC_{90} = 1 mg/mL), had some activity against vancomycin-resistant isolates (MIC_{50} = 1 mg/mL; MIC_{90} = 16 mg/mL) and was less effective against vancomycin susceptible or resistant-*E. faecalis* (MIC_{90} = 32 mg/mL) (95). *In vivo* **55** has shown activity similar to vancomycin in a rabbit model of aortic endocarditis MRSA infection (96), but in similar studies with three organisms of varying susceptibility it was emphasized that the transition from susceptible to resistant strains may occur over a narrow concentration range (97).

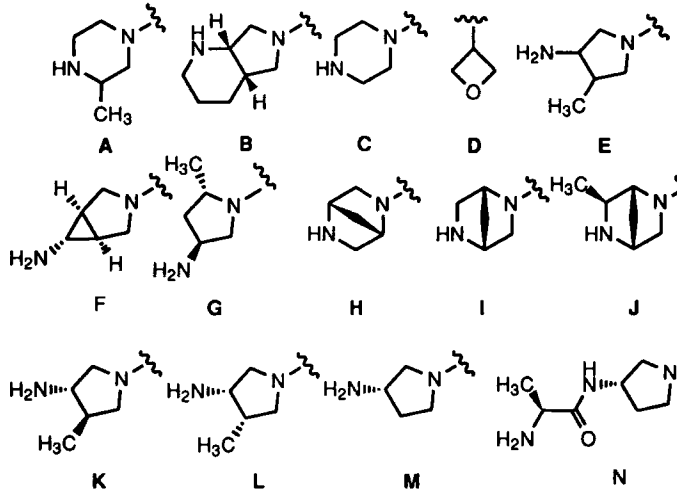
Quinolones - The quinolones remain a major area of interest with research centering on improved activity against gram-positive, particularly MRSA, and ciprofloxacin-resistant organisms, and reduced cytotoxic activity towards eucaryotic cells. Several recent reviews have described the history (98), structure-activity relationships (99-100), and microbiology and clinical usage (101) of the quinolones. A direct correlation was obtained between hydrophobicity as measured by the log of the octanol/pH 7.2 buffer distribution (log *D*) and the intracellular accumulation of eleven quinolones in gram-positive *S. aureus* while two gram-negative organisms, *P. aeruginosa* and *E. coli*, gave an inverse correlation. However, no correlation was observed between intracellular accumulation and MICs strongly suggesting that both intracellular accumulation and intrinsic activity against DNA gyrase are important criteria for antibacterial activity (102). The use of a temperature sensitive topoisomerase II mutant provided strong evidence that the inhibition of topoisomerase II is the physiological target responsible for quinolone cytotoxicity in yeast (103). A systematic study of a wide variety of substituents at the 1,5,7, and 8 positions of the 6-fluoroquinolone/naphthridone nuclei examined the relationships between antibacterial activity against gram-positive and gram-negative organisms and mammalian cell cytotoxicity. Generally, cytotoxicity correlated with increased gram-positive activity, and no single substituent had a controlling effect on the observed cytotoxicity. However, certain trends were apparent including the increased cytotoxicity, and antibacterial activity, of 8-halo quinolones, and, at the 1-position, the difluorophenyl substituent generally led to less cytotoxic quinolones than the corresponding 1-cyclopropyl analogues (104). The recent withdrawal of

temafloxacin, **58**, due to unanticipated adverse drug reactions, including hypoglycemia and hemolytic anemia, may complicate the toxicity issues with future 1-difluorophenyl quinolones (105-106).



	R_1	R_5	R_6	R_7	X
58	DFP[a]	H	F	**A**	CH
59	CP[b]	H	F	**B**	CCl
60	CP[b]	H	F	**C**	CH
61	**D**	H	F	**E**	CCl
62	CP[b]	H	F	**E**	CCl
63	DFP[a]	H	F	**E**	CCl
64	DFP[a]	H	F	**F**	N
65	DFP[a]	H	F	**G**	CH
66	CP[a]	H	F	**H**	CH
67	$C(CH_3)_3$	H	F	**I**	N
68	CP[a]	CH_3	F	**J**	N
69	$C(CH_3)_3$	H	F	**K**	N
70	C(CH3)3	H	F	**L**	N
71	CP[a]	H	F	**M**	N
72	CP[a]	H	F	**N**	N

[a] 2,4-difluorophenyl; [b] cyclopropyl



Several new quinolones were reported for the first time in the past year. BAY Y3118 (**59**) which has the stereochemically defined [S,S] piperidinopyrrolidine substituent at the 7-position (107) exhibited excellent gram-positive activity including MRSA and quinolone-resistant *S. aureus* (32 to 64-fold ciprofloxacin, **60**) and gram-negative activity similar to **60** (108-110). In rats, dogs, and monkeys **59** is nearly completely absorbed orally, and is eliminated mainly as metabolites via the bile (111). In humans **59** had a terminal half-life of 11.4 hrs (112). WQ 1197 (**61**) has a unique N-1 oxetane substituent and while the compound is very similar to **60** in *in vitro* potency and spectrum (113), **61** exhibited much lower cytotoxicity than the corresponding analogues with N-1 cyclopropyl (**62**) or difluorophenyl (**63**) substituents (114). Two new N-1 difluorophenyl quinolones, CP 99,219 (**64**) (115 - 118) and A 80556 (**65**) (119-120) were reported with improved gram-positive activity

relative to **60**. Interestingly, while several strains of anaerobes were not susceptible to **64** (117), **65** had good anaerobe activity (120).

A comparison of the antibacterial activity of a series of isomeric fluoroquinolones indicated that the usual 6-fluoro substituent could be placed at the 8-position, but not the 5-position, and maintain equivalent activity (121). Replacement of the distal amine group on the 7-position pyrrolidine or piperidine substituent with a nonbasic oxime or oxime ether led to compounds with enhanced gram-positive activity but greatly reduced gram-negative activity (122). A systematic study of a large number of diazabicyclo 7-position substutuents led to the selection of danofloxacin (CP 76,136, **66**) for development as a veterinary agent (123). An effort to reduce the histamine-mediated pseudoallergic effects observed with BMY 40062 (**67**) in clinical trials led to the synthesis of a series of methylated 7-(2,5-diazabicyclo[2.2.1] naphthridines) with 1-t-butyl and cyclopropyl substituents. While N-methylation reduced antibacterial activity, the (6S)-6-methyl-2,5-diazabicyclo[2.2.1]-heptane analogue **68** reduced the histamine-mediated hypotension observed for **67** at doses less than 5 mg/kg iv in an anesthetized dog model (124). The synthesis and evaluation of a series of chiral 7-pyrrolonaphthridines led to the conclusion that the S configuration at the amino substituent was optimal and the selection of C-methyl pyrrolidines BMY 41802 (**69**) and BMY 41889 (**70**) for further evaluation (125). A study of amino acid amides of PD 131628 (**71**) revealed that the L-Ala amide (PD 131112, **72**) had improved solubility properties and was completely converted to **71** within 15 minutes after iv or po dosing in rats, dogs, or monkeys, and was greater than 50% converted to **71** by human serum in one hour (126).

ANTIFUNGAL AGENTS

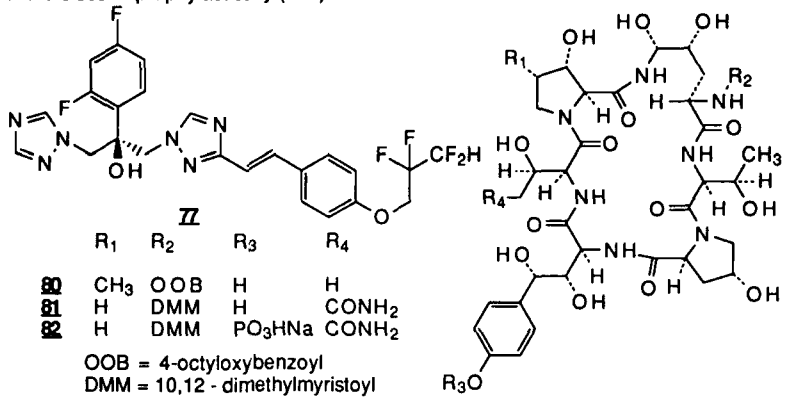
A review of the clinical pharmacology and therapeutic applications of systemic antifungal drugs has appeared (127) as has an examination of antifungal chemotherapy in AIDS patients (128).

Polyenes - Amphotericin B (AmB) remains the gold standard of therapy for deep seated fungal infections, however toxicities, particularly nephrotoxicity, limit its clinical use (129). A comparison of the safety, pharmacokinetics, and drug distribution of AmB colloidal dispersion (ABCD), a stable colloidal complex of amphotericin B and cholesteryl sulfate, and AmB-deoxycholate in dogs indicated that ABCD was eight-fold less toxic. Reduced AmB levels in the kidneys correlated with the reduced toxicity observed in these organs. Up to 90% of the AmB administered as ABCD was recovered from the liver and spleen but no increased toxicity in these organs was observed (130). Generally speaking ABCD had similar *in vitro* activity to AmB-deoxycholate although occasional large differences were noted (131). *In vivo* in a model of disseminated murine cryptococcosis ABCD was equipotent to AmB but greater efficacy could be achieved using four-fold higher doses of ABCD (132). Reduction of the 16-carboxyl of AmB to a hydroxymethyl (133) afforded BRL 49594A (**73**) which exhibited a greater selectivity for lysing fungal cells as opposed to erythrocytes (134). Compared to AmB, **73** had similar *in vivo* antifungal activity in murine models of *Candida albicans* and *Aspergillus fumigatus*, and was at least ten-fold less nephrotoxic in a two week toxicity test in rats (135). An evaluation of MS 8209 (**74**), the N-methyl glucamine salt of 1-deoxy-1-amino-4,6-O-benzylidene-D-fructosyl-AmB, indicated that **74** was as or more effective than AmB in murine protection models against *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* challenges when both compounds were administered at their maximum tolerated doses (15 mg/kg for **74**; 0.5 mg/kg for AmB) (136).

Azoles - A study of itraconazole (**75**) therapy showed that **75** is highly effective for the treatment of blastomycosis and histoplasmosis (137). The oral bioavailability of **75** and saperconazole (**76**) was improved by complexation with cyclodextrins in mice (138). A new triazole, D 0870 (**77**)(139), the R-enantiomer of ICI 195,739 (**78**), showed good efficacy in murine models of *C. albicans* (140) and *C. neoformans* (141) infection compared to fluconazole **79** but was less effective than **75** in an *A. fumigatus* model (142).

Lipopeptides - Efforts in the lipopeptide area have concentrated on expanding the therapeutic spectrum, improving water solubility, and exploring structure-activity relationships. The chemistry and SAR of the echinocandin lipopeptides has been reviewed (143). After initial reports of limited efficacy in an *A. fumigatus* infection model with cilofungin (**80**) (144), subsequent reports have established the inhibition of *Aspergillus* 1,3-β-D-glucan synthase with both **80** (145) and L 688,786 (pneumocandin B_0, **81**) (146). The synthesis of a number of water-soluble esters of **81** led to the discovery of the phosphate prodrug L 693,989 (**82**) (147). In a rat model of *Pneumocystis carinii* infection, **81** and **82** were more effective than **80** in reducing the cyst load, but had little effect on

the number of trophozoites as measured by a DNA probe. However, **82** was able to prevent the onset of the disease prophylactically (148).



77

	R_1	R_2	R_3	R_4
80	CH_3	OOB	H	H
81	H	DMM	H	$CONH_2$
82	H	DMM	PO_3HNa	$CONH_2$

OOB = 4-octyloxybenzoyl
DMM = 10,12 - dimethylmyristoyl

Preparation of a number of simplified analogues of **80** by total synthesis revealed that the presence of a homotyrosine, as opposed to a tyrosine residue was critical for anti-candidal activity and glucan synthesis inhibition *in vitro* (149). The isolation of a number of minor pneumocandins, including **81** (150), along with synthetic methodology for the selective reduction of the ionizable hemiaminal and benzylic positions (151) allowed a comparison of the anti-*Pneumocystis* and anti-*Candida* activities of a number of analogues differing in the extent and position of hydroxylation (152). Generally speaking compounds in which the hemiaminal was reduced had somewhat less *in vivo* efficacy in a *C. albicans* infection model, but similar efficacies in the rat *P. carinii* model.

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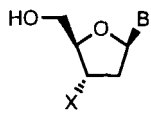
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Chapter 14. Antiviral Agents

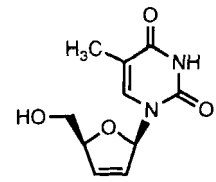
Joel C. Barrish and Robert Zahler
Bristol-Myers Squibb Pharmaceutical Research Institute
Princeton, NJ 08543-4000

Introduction - This chapter will provide an overview of developments in the antiviral field over the last year with emphasis on new chemical entities, utilities, and clinical studies. The principal focus of antiviral chemotherapy research continues to be the development of agents that inhibit the replication of human immunodeficiency virus (HIV), the etiological agent of the acquired immunodeficiency syndrome (AIDS). New agents for the treatment of herpes, hepatitis, influenza, and rhinovirus infections will also be reviewed.

Human Immunodeficiency Viruses - Zidovudine (AZT, **1**), approved by the Food and Drug Administration (FDA) in 1987, remains the primary treatment for HIV infection (1). The results of a recent clinical trial support the hypothesis that early treatment of HIV-1 infection with AZT, combined with *Pneumocystis carinii* pneumonia prophylaxis, improves survival and slows progression to AIDS (2). The development of resistance to AZT upon prolonged therapy has provided impetus for the development of alternative therapies (3). In the fall of 1991, didanosine (ddI, **2**) was approved by the FDA for the treatment of persons in whom AZT treatment had failed or who had become intolerant to AZT (4). In a recent study involving patients who had tolerated AZT for at least 16 weeks, the authors concluded that changing treatment from AZT to ddI appears to slow the progress of HIV disease (5). Based on the results of this study, the indications for ddI were expanded to include adult patients with advanced HIV infection who have received prolonged prior AZT therapy. Also, this year zalcitabine (ddC, **3**) was approved for use in combination with AZT for HIV infection (6). The approved indication was based on data from two small studies in which patients treated with combination therapy had greater CD4+ cell response than those who received AZT alone (7,8). In phase I/II clinical studies, stavudine (D4T, **5**) was well tolerated at doses less than 2 mg/kg/day. Improvements in CD4+ counts, weight gain, and general well-being were seen. Patients on D4T therapy who responded with a 50% improvement in CD4+ cell counts maintained improved counts for a median duration of >1 year (9,10).



	X	B
1	N_3	T
2	H	Hx
3	H	C
4	H	G

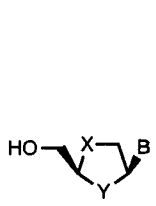


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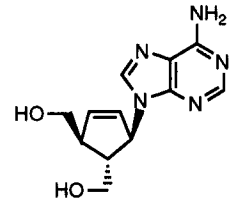
Nucleoside analogs containing novel dideoxy- or deoxyribose surrogates have recently received considerable attention. The enantiomers of 2',3'-dideoxy-3'-thiacytidine (3TC) and its 5-fluoro analog (FTC) were synthesized and evaluated for activity against HIV-1 and -2. The L-(-)-enantiomers of 3TC (**6**) and FTC (**7**) are more potent and less cytotoxic than the corresponding D-(+)-enantiomers (11-19), and L-(-)-3TC has entered phase I/II clinical trials (20,21). In contrast, although L-(-)-2',3'-dideoxy-3'-oxacytidine (dioxolane-C, **8**) is more active than the corresponding D-(+)enantiomer, it is also more cytotoxic (22-24). Both enantiomers of isonucleoside **9** have anti-HIV activity (25-27), and the "deoxyribose" nucleoside analog (-)-(1R,4S,5R)-BCA (**10**) is active against HIV-1, while its enantiomer is devoid of activity (28,29).

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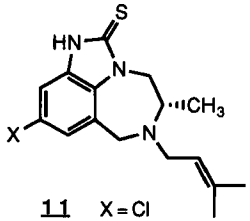
	X	Y	B
6	S	O	C
7	S	O	5-fluoro-C
8	O	O	C
9	O	CH_2	A



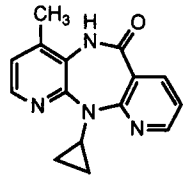
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The clinical usefulness of the currently approved nucleoside analogs is counterbalanced by their toxic side-effects. In addition, structurally and mechanistically diverse drugs used in combination may prevent or delay resistance development during AIDS chemotherapy (3,4,8). These factors have stimulated the search for new and selective agents with alternative modes-of-action. Over the past three years, several classes of non-nucleoside reverse transcriptase (RT) inhibitors have been identified with potent activity against HIV-1 (including AZT-resistant HIV-1) but not against HIV-2 and other viruses (30-32). Despite their structural heterogeneity, these compounds share certain mechanistic features. All are selective inhibitors of HIV-1 RT and display little if any activity against other retroviral or host polymerases. Furthermore, unlike the nucleoside analogs, these compounds bind to the enzyme at a site distinct from the substrate binding site without the need for prior metabolic activation.

R-82913 (11) and R-82150 (12) are potent tetrahydroimidazo-benzodiazepinone (TIBO) inhibitors of HIV-1 *in vitro* (IC_{50} = 26 and 29 nM, respectively) (33). Phase I clinical studies demonstrate that 11 is well tolerated upon *i.v.* administration; however, the oral bioavailability of 11 is low (7-10%) (34,35). Nevirapine (BI-RG-587, 13) is a structurally distinct non-nucleoside RT inhibitor that seems to bind to the same site as 12 (36). The nevirapine binding site, a deep pocket between the "palm" and "thumb" subdomains of the p66 pol subunit, has been identified by photoaffinity labeling and X-ray crystallography (37,38). The human pharmacokinetics of nevirapine, either alone or in combination with AZT, is compatible with once-a-day oral dosing, resulting in steady state plasma concentrations significantly higher than those required to inhibit HIV-1 replication in cell culture (39).

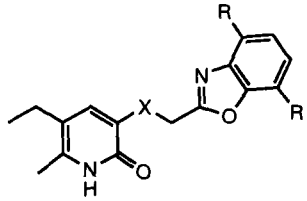


11 X=Cl
12 X=H

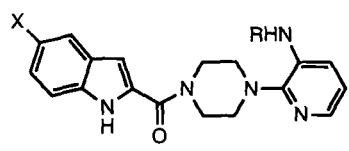


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L-697,639 (14) and L-697,661 (15) show synergy with both AZT and ddI and are active against AZT-resistant HIV-1 isolates *in vitro* (40,41). These pyridones appear to inhibit RT activity by the same mechanism as 12 and nevirapine. L-696,229 (16) is related structurally to 14 and 15 but contains a metabolically stable ethylene linker and is devoid of substituents on the benzoxazole ring. Pyridone 16 prevents the spread of HIV-1 infection at concentrations similar to 14 and 15 and binds less to human plasma (42-44). The oral bioavailability of 16 in rats and monkeys is 7-8% for both species and the plasma half-lives are 25 and 41 min., respectively (45). The bisheteroarylpiperazine (BHAP) RT inhibitor U-87201 (17) blocks HIV-1 replication in a SCID-hu mouse model when administered orally beginning 1 day before *i.v.* infection and continuing for 14 days (46). Early clinical trials suggest that 17 is orally bioavailable and well tolerated (47-49). Unlike 17, the more potent analog, U-88204 (18), completely blocks the spread of virus from infected to uninfected MT-2 cells (50). U-88204 competes with nevirapine for binding to RT (51).

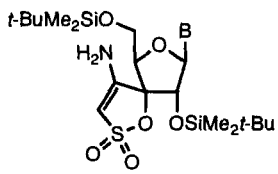


	X	B
14	NH	CH_3
15	NH	Cl
16	CH_2	H

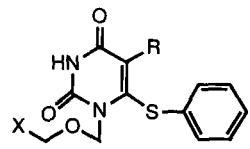


	X	B
17	OCH_3	CH_2CH_3
18	H	$CH(CH_3)_2$

Although the TSAO (19) (52-56) and HEPT (20 - 21) (57-61) classes of compounds contain structural elements in common with nucleosides, they exert their anti-HIV activity as non-nucleoside RT inhibitors. TSAO-T is a potent inhibitor of HIV-1 replication, including strains resistant to AZT. TSAO-3-Me-T is particularly active (IC_{50} = 0.059 uM) and selective (CC_{50} = 240 μM). Although HEPT itself (20) is a weak inhibitor of HIV-1 in cell culture (IC_{50} = 6.5 μM), structure-activity studies have provided a variety of promising analogs, including E-EPU (21) (IC_{50} = 0.022 μM, CC_{50} = 146 μM).



19 B = T, 3-Me-T



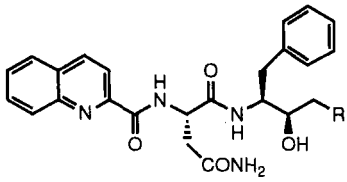
	B	X
20	CH_3	CH_2OH
21	CH_2CH_3	CH_3

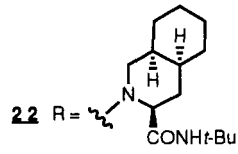
It had been recognized early on that the exquisite selectivity of the non-nucleoside RT inhibitors for HIV-1 over HIV-2 might portend rapid resistance development (62). Mutations might be tolerated at the binding site of a non-nucleoside RT inhibitor that would permit replication but render the virus resistant to drug. In fact, serial passage in cell culture of HIV-1 in the presence of several inhibitors results in the rapid emergence of resistant variants (50,63-65). Until recently, it was assumed that HIV-1 mutant strains resistant to one class of non-nucleoside RT inhibitor would also be cross resistant to other classes. For example, HIV variants resistant to nevirapine and 15 have a substitution of cysteine for tyrosine at RT position 181, and this amino acid change results in a significant reduction of sensitivity to all classes of non-nucleoside RT inhibitors. However, a recent report shows that different non-nucleoside RT inhibitors select for different amino acid changes conferring varying degrees of cross resistance to other non-nucleoside inhibitors (66). For example, some TSAO- and TIBO-resistant strains can maintain sensitivity to nevaripine and 15. In recent clinical trials with nevirapine and 15, significant resistance to each agent developed rapidly (67,68). Although these observations are discouraging for monotherapy, higher doses may overcome resistance, and the non-nucleoside RT inhibitors may find clinical utility in combined chemotherapy.

Drugs which intervene late in the HIV replication cycle will be an important addition to the clinician's armamentarium for the treatment of AIDS. One such target, HIV protease, has attracted considerable attention due to its critical role in the production of mature infectious virus (69-71). The identification of HIV protease as an aspartyl protease, along with a wealth of structural

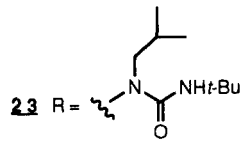
information, has provided impetus for medicinal chemists to design transition-state analog peptide-mimetics (72,73).

Ro 31-8959 (**22**) is a potent and selective inhibitor of HIV protease ($K_i \cong 0.1$ nM) with excellent antiviral activity against several strains of HIV-1 and -2 (IC_{50} = 1-10 nM) (74). Consistent with observed structure-activity relationships, an X-ray crystal structure of Ro 31-8959 complexed with HIV-1 protease shows that the inhibitor is tightly bound to the enzyme in the region of the decahydronaphthalene group (75). Although resistant variants have been generated in cell culture, the rate of resistance development is slower than that observed for the non-nucleoside RT inhibitors and AZT (76). Phase I clinical studies with Ro 31-8959 have demonstrated that, despite its low oral bioavailability (ca. 4%), a dosage regimen of 600 mg three times daily produces plasma concentrations significantly greater than the IC_{90} throughout the dosing interval (77-79). Additive to synergistic activity was seen with Ro 31-8959 combined with AZT, ddC, or IFN-α against AZT-sensitive and AZT-resistant HIV-1 isolates without additive toxicity (80). These results are encouraging for the use of Ro 31-8959 in combination with other antiviral agents. A closely related inhibitor, SC-52151 [**23**, IC_{50} (HIV-1) ≅ 40 nM], was reported to act synergistically with AZT and ddI *in vitro* and have 17% oral bioavailability in rats (81).



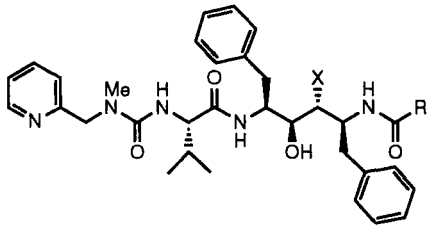


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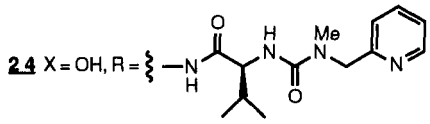


23 R =

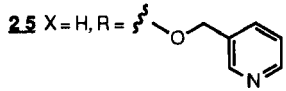
C_2 symmetric inhibitors complement the C_2 symmetry of the HIV protease binding site and represent one of the more ingenious approaches to the rational design of HIV protease inhibitors. A77003 (**24**), a C_2 symmetric inhibitor, exhibits broad-spectrum activity against several strains of HIV-1 and -2 (including clinical isolates and AZT-resistant HIV) in a variety of cell lines (IC_{50} = 0.03 - 0.3 μM) (82,83). There is a strong trend toward synergistic antiviral activity when A77003 is combined with AZT (84). A77003 has substantially improved water solubility than earlier lead compounds and is in phase I clinical trials as a potential *i.v.* drug (85). A truncated, non-C_2 symmetric analog, A-80987 [**25**, IC_{50}(HIV-1) = 0.13 μM], is reported to have oral bioavailability ranging from 13-26% in the rat, dog and monkey without formulation and >50% in the dog when formulated (86,87). Using an *in vitro* plaque assay, HIV-1 variants have been isolated with 8-fold reduced sensitivity to C_2 symmetric diol inhibitors and no significant cross resistance with **22** (88). A single point mutation of valine 82 to alanine was the cause of the reduced sensitivity (89).



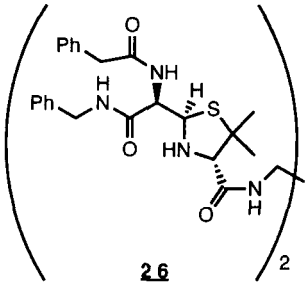
24 X = OH, R =



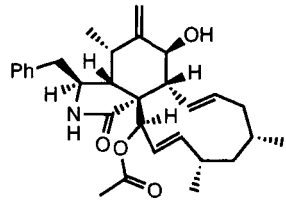
25 X = H, R =



Several companies are using high-throughput screens in an attempt to find novel inhibitors of HIV protease. The penicillin-derived C_2 symmetric dimer **26** was identified as a potent and selective inhibitor of HIV protease (IC_{50} = 0.9 nM) with good activity against HIV-1 (IC_{50} = 0.29 μM) (90). An x-ray crystal structure has shown that **26** binds symmetrically to the enzyme. L-696,474 (**27**), a novel cytochalasin isolated from *Hypoxylon fragiforme*, is a competitive inhibitor of HIV-1 protease (apparent K_i = 1 μM) (91-93).

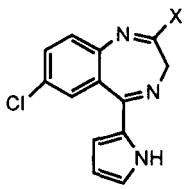


26

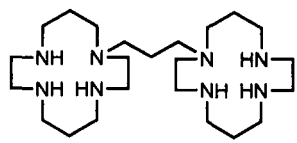


27

Among the regulatory gene products encoded by HIV-1 is a strong positive regulator, Tat. It is required for HIV-1 replication, has no apparent cellular homologs, and is an attractive target because inhibition would arrest virus replication at an early stage. Ro 5-3335 (**28**) was identified in a random screen as a Tat inhibitor (94-97). Consistent with its mode of action, Ro 5-3335 inhibits HIV replication in chronically infected cells. When tested against acute infection, Ro 5-3335 is active against a spectrum of HIV strains, including HIV-2 and AZT-resistant HIV-1. The anti-HIV activity, cytotoxicity, and selectivity index of Ro 5-3335 is highly dependent on the host cell, and the mechanism of action of Ro 5-3335 seems to involve at least one cellular component (98). Although Ro 5-3335 displays considerable (85%) oral bioavailability in dogs, a substantial accumulation of a hydroxypyrrole metabolite may be responsible for kidney damage observed at high doses in rats (99). A second generation benzodiazepine, Ro 24-7429 (**29**), has *in vitro* properties similar to Ro 5-3335 and is in phase I/II clinical trials (85, 100,101).



28 X = OH
29 X = $NHCH_3$



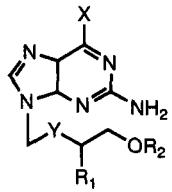
30

JM2763 (**30**) is a novel macrocyclic polyamine (a "bicyclam") with broad spectrum activity against HIV-1 and HIV-2 in several T-cell systems (IC_{50} = 0.12-1.4 μM) and little cytotoxicity. Mechanism of action studies reveal that **30** interacts at an early event in the viral replicative cycle, tentatively identified as a viral uncoating event (102).

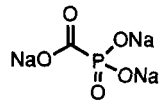
Herpesviruses - Acyclovir (**31**) remains the treatment of choice for primary and recurrent herpes simplex and herpes zoster (shingles) infections. Two recent clinical trials demonstrate that acyclovir is both a safe and effective treatment for varicella (chickenpox) in otherwise healthy children and adolescents (103,104). Based on these studies, the indications for acyclovir have been expanded to include chickenpox in normal children and adolescents (105).

In September, 1991, foscarnet (**36**) became the second antiviral agent to be approved by the FDA for the therapy of AIDS-related cytomegalovirus (CMV) retinitis (106). In a clinical trial designed to compare foscarnet with ganciclovir (**32**) for the treatment of CMV retinitis in AIDS patients, foscarnet offered a survival advantage over treatment with ganciclovir (107). However, there was no difference between the two groups in the rate of progression to retinitis, and patients may not tolerate foscarnet as well as ganciclovir. Foscarnet is clinically effective against acyclovir-resistant herpes infections in AIDS patients (108). A supplemental New Drug Application has

filed to expand the approved indications for foscarnet to include treatment of acyclovir-resistant *herpes simplex virus* (HSV) type 1 and 2 infections (109).



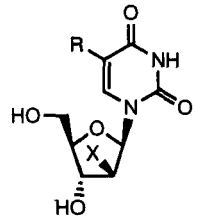
	X	Y	R_1	R_2
31	OH	O	H	H
32	OH	O	CH_2OH	H
33	OH	O	H	L-valyl
34	OH	CH_2	CH_2OH	H
35	H	CH_2	CH_2OAc	Ac



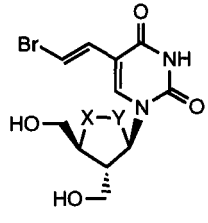
36

The oral bioavailabilty of acyclovir in humans is only 15-20%. The L-valyl ester of acyclovir (BW 256U87, **33**) is 3-fold better absorbed than acyclovir upon oral administration and is now undergoing clinical evaluation (110,111). Penciclovir (**34**) is a selective agent with promising *in vitro* activity against HSV and varicella-zoster virus (VZV) (112,113). A recent clinical trial comparing penciclovir and acyclovir for the *i.v.* treatment of herpes zoster in immunocompetent patients shows both agents to be equally efficacious (114). Although the oral bioavailability of penciclovir is very poor (115), clinical trials indicate that famciclovir (**35**) functions effectively as a penciclovir prodrug, with 60% of the oral dose recovered as penciclovir in the urine (116).

BVaraU (sorivudine, **37**) is a potent (IC_{50} = 0.001 μM) and highly selective inhibitor of VZV *in vitro* (117). BVaraU administered p.o. twice daily to monkeys at 0.1 mg/kg for 10 days following infection with simian varicella virus prevented vesicular rash development and suppressed viremia (118). The oral bioavailability and serum half-life of BVaraU in man was recently reported to be 60% and 6 h, respectively (119). PYaraU (**38**) is less potent than BVaraU against VZV (IC_{50} = 0.6-4 μM), and its oral bioavailability and serum half-life in man are 20% and 12 h (120,121). Two bromovinyuracil nucleoside analogs with novel 2'-deoxyribose surrogates, BMS 181,165 (**40**) and SQ 35,452 (**41**), were reported to have potent and selective activities against VZV (0.1-0.3 and 0.02-0.2 μM, respectively) (122,123).



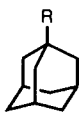
	X	R
37	OH	
38	OH	$-C\equiv CCH_3$
39	F	I



	X	Y
40	O	CH_2
41	$-CH_2-$	

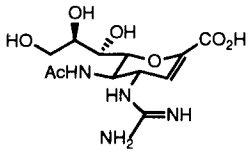
Hepatitis B Virus - Although there are distinct differences between HIV and hepatitis B virus (HBV), the replicative cycle for both viruses involves an obligative reverse transcription step. Several groups have pursued the experimental therapy of HBV infection by targeting the viral reverse transcriptase using dideoxynucleoside analogs such as 2',3'-dideoxyguanosine (ddG, **4**), ddC (**3**), L-(-)-3TC (**6**), and L-(-)-FTC (**7**) (124-128). Recently, the 2'-deoxynucleoside analog FIAU (**39**) was tested in a small clinical trial on patients with chronic HBV and concomitant HIV infection. The results suggest that FIAU is efficacious in patients with baseline DNA levels below 600 pg/ml. Common side effects include nausea, elevated hepatic transaminases, and fatigue (129).

Influenza Viruses - Amantadine (**42**) and rimantadine (**43**) are specific anti-influenza A drugs. Both agents block virus replication after the virus has bound to the cell but before uncoating occurs. Nucleotide sequence analysis of drug-resistant mutants shows that resistance results from single amino acid changes in the transmembrane region of the influenza A virus M2 protein (130,131). A recent study in which the M2 protein was expressed in Xenopus laevis oocytes provides strong evidence that the M2 protein functions as a proton channel, and that the adamantanamines inhibit virus replication by blocking that channel, thereby interfering with virus uncoating (132).



42 R = NH_2

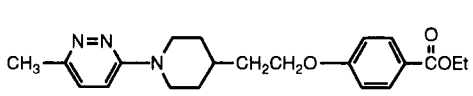
43 R = $CH(NH_2)CH_3$



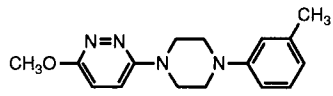
44

The potent and selective influenza neuraminidase inhibitor 4-guanidino Neu 5Ac2en (**44**) [K_i(viral) = 0.1 nM; K_i(human) = 100 μM)] inhibits the replication of both influenza A and B viruses in cell culture and is efficacious in animal models when administered intranasally (133).

Rhinoviruses - Human rhinoviruses (HRV's) are a major cause of the common cold. A variety of anti-HRV agents have been shown to possess high *in vitro* activity against a selection of HRV serotypes. All of them seem to neutralize rhinoviruses by binding to a specific hydrophobic pocket within the virion capsid protein VP1. Pirodavir (R 77975, **45**) is a novel, capsid-binding anti-picornavirus agent with potent *in vitro* activity against a broad spectrum of HRV serotypes. Although its predecessor, R 61837 (**46**), can reach an IC_{50} value against 80% of the 100 serotypes tested at concentrations above 32 μg/ml, pirodavir inhibits the same percentage at 0.064 μg/ml (134).



45



46

A potentially general and selective approach to antiviral chemotherapy involves the use of synthetic oligonucleotide analogs complementary to viral DNA or RNA to inhibit viral gene expression, i.e., the "antisense" approach. The promise (generality, specificity) and problems (specificity, size, cell penetration, metabolic stability, pharmacokinetics, synthesis) associated with antisense inhibitors have been the subject of several recent reviews (135-137).

Conclusion - The global pandemic of HIV is entering its second decade. Progress has been made toward slowing the course of the disease and treating a host of concomitant opportunistic infections; however, there is no cure for AIDS. It is for these reasons that antiviral research continues to focus primarily on the discovery of agents for the treatment of HIV infection. In 1991 - 1992, two new nucleoside analogs (ddI and ddC) were approved by the FDA for the therapy of HIV, and other nucleoside analogs are in clinical trials. However, the principal focus of AIDS medicinal chemical research has shifted from nucleoside-analog reverse transcriptase inhibitors to agents with alternative modes-of-action, such as inhibitors of HIV protease and Tat, as well as non-nucleoside reverse transcriptase inhibitors. The novel Tat inhibitor Ro 5-3335 and the wide array of structural types found to serve as non-nucleoside RT inhibitors were found by "random" screening of chemical libraries. Viral pathogens encode a limited number of essential proteins and an even smaller number of essential enzymes that are amenable to the "rational design" of low molecular weight inhibitors. Therefore, high throughput screening of both chemical libraries and natural products will continue to play an important role in the future.

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Chapter 15. Recent Developments in Antibacterial Resistance Mechanisms

Katherine E. Brighty, William Kohlbrenner and Paul R. McGuirk
Pfizer, Inc., Central Research Division, Groton, CT 06340

Introduction - The increasing clinical importance of drug-resistant bacterial pathogens has lent additional urgency to microbiological and antibacterial research (1-3). As pointed out in a number of recent reviews, there have been relatively rapid and dramatic changes in the antibiotic resistance patterns in both community and institutional settings, which will likely have considerable impact on therapeutic strategies for the treatment of bacterial infections (4,5). The common respiratory pathogens *Streptococcus pneumoniae* (6), *Haemophilus influenzae* (5) and *Moraxella catarrhalis* are becoming increasingly resistant to β-lactams. Resistance development also limits utility of macrolides in the long-term treatment of *Mycobacterium avium*, a leading cause of death in HIV-infected patients. Most worrisome as a public health threat is the recent increase in the incidence of tuberculosis caused by multidrug-resistant strains of *Mycobacterium tuberculosis* (7).

A similar limitation of therapeutic options is occurring in the hospital setting, where the percentage of infections caused by drug-resistant gram-positive nosocomial pathogens is increasing. Parenteral vancomycin is now the only major treatment option in many cases of methicillin-resistant *Staphylococcus aureus* (MRSA). Perhaps the most disturbing trend among the gram-positive pathogens has been the increase in serious infections due to enterococci. Currently second only to *Escherichia coli* as a cause of nosocomial infections, enterococci are inherently resistant to most antibiotics, and have historically been treated with synergistic combinations of β-lactams and aminoglycosides. Enterococcal strains have now been isolated, however, which are resistant to β-lactams (8), aminoglycosides (9), and, in some cases, vancomycin (10) -- leaving no effective therapeutic options (11).

The three basic strategies employed by microorganisms in response to the selective pressure of chemotherapy are: 1) alteration in target accessibility, either through decreased permeability or increased efflux of drug; 2) drug modification; and 3) target modification, resulting in reduction of affinity of the target for the drug, or substitution of an alternate pathway carrying out the target function (12,13). The impact of these individual innovations can be magnified by transfer of the relevant genetic information among species and genera by conjugation, transformation, or transduction. This chapter will highlight recent discoveries in mechanisms of resistance to the major classes of antibacterial agents. A detailed understanding of these mechanisms, unfortunately not yet available, will aid in development of new antibacterial agents with which to combat the increasing threat of drug-resistant pathogens.

Fluoroquinolones - Although it was anticipated that the fluoroquinolones had the potential to be safe, oral therapy for MRSA, this has proven not to be the case. Rapid development of high-level resistance of MRSA ($MIC_{90} \geq 64$ μg/ml) to ciprofloxacin, in up to 79% of recent isolates, has occurred. Resistance of *Pseudomonas aeruginosa* has also become problematic. In some cases the resistance of *P. aeruginosa* has been as high as 45 to 50%. In addition, high level quinolone resistance has developed against 14% of methicillin-susceptible *S. aureus* (14,15). The mechanisms of resistance to fluoroquinolones have been reviewed recently (16,17). Chromosomal mutations resulting in modifications in critical residues of DNA gyrase and decreased drug permeation have been documented as the predominant mechanisms of resistance to the fluoroquinolones (18). No confirmed cases of plasmid-mediated resistance with clinical consequences have been reported (19).

Several examples of mutations in the A subunit of DNA gyrase leading to high-level resistance to fluoroquinolones in the clinic and subsequent treatment failure have been documented using PCR, DNA sequencing techniques, and plasmid-encoded gene replacement (20-23). In *S. epidermidis*, *S. aureus*, and *S. saprophyticus*, it was shown that mutations bearing Phe or Leu at Ser-84 resulted in resistance to ciprofloxacin (24). In one study of 34 MRSA strains examined, 28 were found to be resistant to ciprofloxacin; all mapped in *gyrA* with 16 containing a Ser-84→Leu mutation, three with Ser-84→Ala, three containing both Ser-84→Leu and Ser-85→Pro, and 6

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strains containing a Glu-88→Lys mutation (25). The Ser-84→Leu mutation conferred the highest levels of resistance, consistent with results for the homologous Ser-83 substitution observed in *E. coli* GyrA (26). Several other studies also confirmed the role of *gyrA* mutations in MRSA quinolone-resistant strains (27,28) and in *K. pneumoniae*, *P. aeruginosa*, *A. calcoaceticus*, and *P. stuartii* (20). Plasmids containing cloned *E. coli* wild-type *gyrA* were used to transform ciprofloxacin-resistant gram-negative clinical isolates to identify strains harboring mutations in *gyrA*. Clinical failure with ciprofloxacin, in a number of infections, was shown to correlate with resistance due to a single mutation in *gyrA* using this method (29). Fluoroquinolone resistance in *C. jejuni* is also due to alterations in *gyrA* (30,31). A mutation at Thr-86→Ile, which is homologous to Ser-83 in *E. coli*, conferred high-level resistance to ciprofloxacin (32).

In one study, tritium-labelled norfloxacin did not bind to a 147 bp fragment of DNA at high drug/DNA ratios (34). Further, no evidence for binding to *E. coli* wild-type gyrase or gyrase with a *gyrA* trp-83 mutation could be demonstrated in the absence of DNA. Significant binding was detected to a complex of wild type gyrase and DNA, however, in agreement with previous results (33). Binding was abolished for complexes of *gyrA* Trp-83 and DNA. This study demonstrated that DNA gyrase, particularly the *gyrA* subunit, plays a significant role in the efficiency of quinolone binding, but DNA is also required.

Alterations in the DNA gyrase B subunit can also affect the susceptibility of bacteria to quinolones. *GyrB* mutants have been observed in clinical isolates of *E. coli*, *N. gonorrhoea* and *P. aeruginosa* (35-37). Quinolone-resistant *gyrB* mutants of *E. coli* KL16 were isolated in the laboratory under selection with nalidixic acid or enoxacin at four times the MIC. Type 1 mutants were resistant to the amphoteric fluoroquinolones and nalidixic acid and type 2 mutants were resistant to nalidixic acid and hypersusceptible to the fluoroquinolones. All of the type 1 mutants had an Asp-426→Asn point mutation while type 2 mutants had a Lys-447→Glu substitution. A model of a potential quinolone binding pocket within GyrB involving the C7 substituent of quinolones was proposed. Changes in net charge at the two mutated sites are used to explain the results with the *gyrB* resistant mutants (38).

Decreased drug accumulation resulting from the reduction or absence of porin channels in the outer membrane and active efflux at the inner membrane also play a role in quinolone resistance. Expression of *ompF* is regulated at both the transcriptional and translational level. Overexpression of *micF*, encoding an antisense RNA that is complementary to the 5'-end of the *ompF* message, presumably reduces *ompF* translation by destabilization of the *ompF* mRNA-ribosome complex. The resistance genes *nfxB*, *nfxC1*, *cfxB1*, and *marA* are also involved in reduced expression of *ompF* and require the *micF* locus (39). Changes in the outer membrane of *P. aeruginosa* have been implicated in altering permeability to quinolones both *via* a decrease in or absence of normal proteins and through expression of unusual additional proteins in response to drug pressure. Of interest is the notion that porin expression is sensitive to the level of DNA supercoiling (40). The outer membrane proteins implicated include a decrease in or loss of OmpF, G, D2, E1, E2, C, and 40 and 46 kDa proteins and an increase in proteins of molecular weight 51 and 54 kDa. The NfxB protein may be a DNA-binding protein that regulates gene expression and negatively regulates the production of outer membrane proteins. It has been shown that mutated NfxB has an arginine replaced by a glycine residue that may lead to the loss of DNA binding and enhanced expression of a 54 kDa outer membrane protein in *P. aeruginosa*. Overproduction of this protein results in decreased permeability to fluoroquinolones and hypersusceptibility to the β-lactams and aminoglycosides (41,42). A clinical isolate of *P. aeruginosa* from a patient treated with enoxacin was cross-resistant to quinolones, β-lactams, chloramphenicol and tetracyclines but not imipenem or aminoglycosides. It was subsequently shown that the strain had lost the OmpF porin and had an altered LPS profile. Interestingly, a single step revertant could be selected that was indistinguishable from the pretreatment isolate, indicating that pleiotropic effects were caused by the same mutation (43). In *P. aeruginosa*, overexpression of the outer membrane protein OprH could be correlated with increased resistance to polymixin B, gentamicin, and EDTA, suggesting that OprH blocks self-promoted uptake of these agents. Surprisingly, overproduction of OprH conferred hypersusceptibility to fluoroquinolones and chloramphenicol. OprH was ruled out as a porin since it failed to show a rise in membrane conductance when added to a lipid bilayer membrane. It was suggested that the resultant increase in uptake is therefore mediated by a non-porin pathway distinct from self-promoted uptake. It was proposed that a general mechanism of quinolone penetration in *P. aeruginosa* could involve an ill-defined LPS pathway (44). In porin-deficient outer membrane mutants of *E. coli*, *P. mirabilis*, and *S. typhimurium*, it was shown that tosufloxacin and sparfloxacin had increased uptake compared to ciprofloxacin, ofloxacin, and norfloxacin. Tosufloxacin and sparfloxacin may be penetrating the outer membrane of these bacteria through a non-porin LPS pathway similar to that described above for *P. aeruginosa*. It is

noteworthy that the ability to penetrate gram-negative bacteria through the non-porin pathway did not correlate with relative hydrophobicity of the quinolones tested (45). An isolate of *P. vulgaris* which was highly resistant to ofloxacin (MIC >800 μg/ml) was shown to possess three types of resistance mechanisms: altered DNA gyrase, absence of a 37 kDa porin protein and an active efflux system not generally seen in gram-negative pathogens (46).

NorA, found in several clinical isolates of *S. aureus*, has been characterized as a chromosomally encoded multidrug membrane-spanning efflux protein that is dependent on a proton gradient and inhibited by protonophores. NorA bears close homology to Bmr in *B. subtilis*, and both proteins are capable of effluxing a variety of drugs of unrelated structure such as rhodamine 6G, ethidium bromide, chloramphenicol, tetraphenylphosphonium bromide, puromycin and acridine orange as well as the quinolones. Both proteins had their activity completely inhibited and their resistance effect on cells completely reversed by reserpine, although approximately 20 times more reserpine was needed to inhibit NorA compared to Bmr (47).

β-Lactams - A number of recent reviews provide detailed surveys of the occurrence and clinical significance of β-lactam resistance (5,48), the role of β-lactamases (49-51) and altered penicillin binding proteins (PBPs) in resistance development (52), and the use of β-lactamase inhibitors (53,54).

Among recent trends is an increase in β-lactam resistance in fastidious gram-negatives such as *N. gonorrhoeae* (55), *H. influenza* (56) and *M. catarrhalis* (57) due to the acquisition of β-lactamase genes. In contrast, *N. meningitidis* (58) and some *N. gonorrhoeae* (55) strains appear to develop resistance as a result of altered PBPs. Much of the plasmid-borne resistance to penicillins and older cephalosporins in *Enterobacteraceae* is due to the TEM-1 and SHV-1 β-lactamases, with the TEM-1 enzyme by far the more prevalent (59). Hyperexpression of these enzymes can also lead to resistance to β-lactamase inhibitor/β-lactam combinations (60), while mutation has led to the appearance of extended broad-spectrum β-lactamases, particularly in *Klebsiella* spp., rendering these organisms resistant to the newer cephalosporins and monobactams (50). In addition, a number of genera, including *Enterobacter, Citrobacter, Serratia* and *Pseudomonas*, encode an inducible chromosomal cephalosporinase. Overproduction of this enzyme frequently occurs due to gene amplification or mutation transcriptional control, leading to resistance to β-lactamase inhibitor/β-lactam combinations, cephalosporins, cephamycins and monobactams (5). β-Lactamase production has also been detected recently in enterococci, important gram-positive nosocomial pathogens (8).

Of note is the emerging resistance to the wide spectrum agent imipenem (61). This compound is generally highly resistant to β-lactamases, however, most strains of *Xanthomonas* (*Pseudomonas*) *maltophilia*, an emerging nosocomial pathogen, are resistant to imipenem due to the expression of a chromosomal carbapenemase (62). Nontransferable carbapenemases have also been detected in a few isolates of *B. fragilis, E. cloacae,* and *S. marcescens* (63,64). Imipenem resistance in *E. cloacae* can also result from overexpression of the chromosomal β-lactamase when combined with mutations leading to reduced outer membrane permeability (65,66). Also of concern is a plasmid-encoded β-lactamase recently discovered in Japan which confers imipenem resistance to *P. aeruginosa* (67). Permeability is also a factor in *P. aeruginosa* susceptibility, since loss of the D2 porin results in imipenem resistance (68,69). The carbapenemases have recently been reviewed (70).

Resistance to β-lactam antibiotics can also develop through the production of altered penicillin binding proteins (71,52). Penicillin-resistant *S. pneumoniae* strains have low-affinity PBPs which demonstrate an altered penicillin binding profile when compared to the PBP profile of susceptible strains (72). There are also recent reports of *S. pneumoniae* strains that are resistant to third generation cephalosporins due to altered PBPs (73,74). Similarly, methicillin resistance in staphylococci is due to PBP 2a (75,76), an atypical PBP that has a low affinity for methicillin and other β-lactams and is encoded by an acquired chromosomal resistance gene (*mec*) (77). Similar *mec* genes have been found in other coagulase-positive and -negative staphylococci (78). Site-specific mutagenesis of an MRSA *mecA* gene provided a water-soluble form of PBP 2a; crystallization and structural analysis of this protein will be invaluable in the design of new agents (79). It should be noted that methicillin resistance in *S. aureus* which involves production of altered PBPs other than PBP 2a has also been reported (80).

Macrolides, Lincosamides and Streptogramins - Numerous mechanisms of macrolide resistance have been discovered (81-83). Target modification, often plasmid- or transposon-mediated, remains the primary mechanism of clinically important resistance to macrolide, lincosamide and streptogramin B-type (MLS) agents. It is observed in a wide variety of gram-positive and gram-negative organisms, including *Streptomyces* strains such as those that produce macrolide

antibiotics (82,84). MLS resistance is effected through production of a variety of related methyl transferases which dimethylate adenine 2058 (*E. coli* numbering) in 23S ribosomal RNA, decreasing the affinity of the ribosome for this structurally diverse group of antibacterial agents. Although a handful of macrolide analogues have been reported to exhibit some activity against MLS-resistant bacterial pathogens, the exact nature of antibiotic binding and how it is affected by adenine dimethylation is still not completely understood (85,86). Data from a mutagenized *E. coli* ribosome containing a single base change in the 23 S rRNA support the hypothesis that ribosomal alterations modulate the conformation of antibiotic binding sites, rather than directly interfering with binding (87). The methylase enzyme can be expressed constitutively or inducibly. In staphylococci, induction is effected through exposure to fourteen and fifteen-membered macrolides, while in streptococci, macrolides, lincosamides and type B streptogramins can all serve to induce MLS resistance. The mechanism of methylase induction is generally one of translational attenuation (88). A recent report describes an example of transcriptional attenuation (89), but it should be noted that subsequent examination of the regulation of a closely related gene provided evidence only for translational attenuation (90). An investigation of erythromycin-resistant clinical isolates of *Campylobacter jejuni* and *C. coli* suggests that a ribosomal protein modification is responsible for the chromosomally mediated, constitutive resistance in these strains (91).

A recently discovered resistance mechanism involves active efflux of fourteen- and fifteen-membered macrolides through utilization of the membrane proton motive force in *Staphylococcus epidermidis* (92). An efflux system specific for fourteen-membered macrolides and type B streptogramins has also been described in *S. epidermidis* (93). In this case, the deduced sequence of the protein responsible for resistance (MsrA) was found to possess two regions homologous to the ATP-binding domains of other transport proteins, leading to the hypothesis that MsrA acts as an ATP-dependent efflux pump. Although this phenotype (termed MS resistance) has historically been observed infrequently in the clinic, a recent study found that one-third of erythromycin-resistant coagulase-negative staphyloccal isolates harbored very similar *msrA* genes. It was noted that the skin may act as a reservoir of MS-resistant strains (94). Homologies to ATP-binding transport proteins were also discovered in three macrolide-resistance encoding genes from macrolide-producing *Streptomyces* organisms (95), and in the staphylococcal plasmid-encoded gene *vga*, which confers resistance to streptogramin A-type compounds (96). The intrinsic resistance of gram-negative bacilli to macrolides results from the inability of classical macrolides to effectively penetrate the outer membrane at achievable serum concentrations. The improved activity of azithromycin over erythromycin against *E. coli* has been attributed to its more effective interaction with the self-promoted uptake pathway due to the presence of an additional basic nitrogen (97).

Molecular modification leading to inactivation of macrolides has also been demonstrated in a number of organisms; however, this mechanism of resistance has not been shown to be of clinical importance (83). Enzymes have been discovered which carry out lactone hydrolysis in clinical isolates of *Enterobacteriaceae* (98,99), glycosylation at the 2'-hydroxyl of macrolide saccharides in *Streptomyces lividans* (100) and phosphorylation at the 2'-hydroxyl in a clinical strain of *E. coli* (101).

Glycopeptides - Vancomycin is presently the only glycopeptide in clinical use worldwide for the treatment of serious infections caused by multiresistant gram-positive bacteria. It comes from a class of compounds that inhibits peptidoglycan synthesis by complexing with peptidyl-D-Ala-D-Ala precursors involved in cell wall assembly (102,103). NMR studies have identified a number of specific hydrogen bonds in complexes of vancomycin or ristocetin with N-acetyl-D-Ala-D-Ala that account for high affinity complex formation (102). Resistance to vancomycin has emerged only recently, in coagulase-negative *Staphylococcus* spp. (104-107), and *Enterococcus* spp. (108-111), in spite of being used for over thirty years. The molecular basis for vancomycin resistance has been best characterized for *Enterococcus* spp., where diverse resistance phenotypes have been observed (109). VanA is a high-level resistance phenotype which is inducible by, and confers cross resistance to, other glycopeptides. VanB confers low-level resistance to all glycopeptides but is inducible only by vancomycin. VanC is a constitutively expressed, low-level resistance phenotype that is thus far specific to *E. gallinarium* (108).

Studies by several groups on the inducible high-level vancomycin resistance in *E. faecium* have revealed an interesting mechanism which confers high-level resistance through the alteration of the peptidoglycan structures to which vancomycin binds (112). This inducible resistance phenotype results from the expression of five plasmid encoded genes: *van*R and *van*S encode the VanR-VanS two-component regulatory system that responds to external vancomycin levels and controls the expression of *van*H (dehydrogenase), *van*A (ligase), and *van*X (unknown function, but required for resistance). VanA is a 38-40 kDa membrane protein that is expressed in large amounts

after induction by vancomycin. This enzyme shares homology with other bacterial D-Ala-D-Ala ligases; however, it has an extended substrate specificity and it can synthesize a number of D-Ala-D-X depsipeptides (where X is a hydroxy acid) that can be incorporated into the cell wall in place of the D-Ala-D-Ala precursors. The VanH dehydrogenase is a 35 kDa protein with D-specific α-ketoacid reductase activity that provides D-2-hydroxy substrates (D-lactate and D-hydroxybutyrate) for VanA. Substitution of the D-Ala-D-hydroxycarboxylic acid moiety for D-Ala-D-Ala results in a 1000-fold decrease in the binding affinity of vancomycin to these altered structures. Recent studies suggest that the substrate used *in vivo* is D-lactate (113). Two other genes, *vanY* (DD-carboxypeptidase) and *vanZ* (encodes protein of unknown function), have been identified as part of this gene cluster but they do not appear to be essential for resistance development (112).

Although less is known about the VanB and VanC phenotypes, there are some apparent similarities with VanA. VanB is a 39.5 kDa protein that shows amino acid homology with other D-Ala-D-Ala ligases (114). The gene encoding VanC has been cloned from *E. galinarium*. The translated amino acid sequence showed significant homology with VanA, suggesting that it also had D-Ala-D-Ala ligase activity. Interestingly, VanC could not substitute for VanA in VanA-deficient strains (115). Inducible resistance to high levels of glycopeptides is transferred by a variety of self-transferable plasmids which contain sequences related to *vanA*. A recent report suggests that in many cases the *van* resistance gene clusters are contained in transposable elements related to Tn3 (116), which facilitate the transfer of this resistance determinant among *Enterococcus* spp.

Tetracyclines - The understanding of bacterial resistance to tetracycline antibacterials has advanced to some degree over the past several years, although many of the molecular details remain obscure. Three mechanisms of resistance to tetracyclines (active efflux, ribosomal protection and inactivation through drug modification) have been identified and recently reviewed (117,118). All three mechanisms have been observed in clinical isolates, the most prevalent being ribosomal protection. Active efflux mechanisms of resistance as they pertain to tetracyclines, fluoroquinolones, macrolides, heavy metals, and antiparasitics were recently reviewed (13). Resistance genes are assigned to a specific class based on DNA-DNA hybridization within members of that class. The different classes have been given letter designations, with related mechanisms grouped together in the alphabet. Generally, efflux determinants A-E, which are located on plasmids, are found among the Enterobacteriaceae and other gram-negative aerobic bacteria. Efflux determinants K and L are found on plasmids in gram-positive organisms; determinant P is found only in *Clostridium*. The ribosomal protection determinants M and O, both plasmid- and chromosomally-encoded, are found among gram-positive, gram-negative and cell wall-less organisms such as *Mycoplasma* and *Ureaplasma* (119). Ribosomal protection determinant Q is found only in *Bacteroides* (120). Occasionally, pathogenic strains harboring both active efflux and ribosomal protection mechanisms of resistance are found (121): *Staphylococcus* isolates expressing both Tet(K) and Tet(M) showed additive effects (122).

The efflux protein is an energy-dependent membrane-bound transporter which ejects a magnesium-chelated form of tetracycline in exchange for a proton (123). It is not known how the efflux proteins function in pumping tetracyclines out of the cell, and elucidation of the exact molecular mechanism is made difficult by the fact that these efflux proteins are highly membrane-bound, containing twelve membrane-spanning regions. Through site-directed mutagenesis of a class B protein, Levy has shown that replacement of aspartate-15 by tyrosine, histidine or asparagine results in a 60 to 85% loss of tetracycline resistance (124). Interestingly, Asp-15 is one of four highly conserved charged residues believed to be embedded in the cytoplasmic membrane. Yamaguchi *et al.* have shown that histidine-257 of the TetA protein in *E. coli* plays an essential role in proton translocation during tetracycline efflux (125). Recently, multidrug-resistant leukemia cell lines were shown to be more resistant to the cytotoxic effects of tetracycline than parental cells. This resistance was reversed by the addition of the known P-glycoprotein efflux pump inhibitors verapamil and cyclosporin A. The use of such an approach in bacteria to overcome resistance due to efflux was recently exemplified (126). Photoaffinity labelling studies revealed a 170 kDa membrane protein in the resistant cell line but not in parental cells. This is the first report suggesting that a hydrophilic drug such as tetracycline is effluxed by P-glycoprotein (127).

The most common resistance mechanism to tetracyclines is ribosomal protection. The proteins responsible for this mechanism are designated Tet(M), Tet(O), and Tet(Q). Tet(M) is found in gram-negative, gram-positive, anaerobes, and cell wall-less bacteria. Tet(O), originally discovered in *Campylobacter* spp., has now been found in *Streptococcus*, *Enterococcus*, and *Lactobacillus*. To date, Tet(Q) has been found only in *Bacteroides* spp. The protein conferring ribosomal protection has a molecular weight of 72 kDa by sequence analysis (128) and 68 kDa by SDS-polyacrylamide gel electrophoresis (129) and associates with the ribosome, allowing protein

synthesis to proceed in the presence of bound tetracycline. The mechanism by which this is accomplished remains obscure. Since only small amounts of Tet(O) or Tet(M) are required to render ribosomes resistant, it is thought that a catalytic mechanism must be involved in which the ribosome becomes transiently altered in a way that allows protein synthesis to continue in the presence of tetracycline, although there is no direct evidence to support this hypothesis (130). Tet(M) and Tet(O) are 75% homologous whereas Tet(Q) is more distantly related, showing only 40% homology to either Tet(M) or Tet(O) (120). These three proteins share homology with elongation factor G (EF-G), particularly in the region containing the GTP-binding site. Tet(M) and Tet(O) have been shown to possess ribosome-dependent GTPase activity, but cannot substitute for EF-G (129,130).

A novel tetracycline resistance determinant was found on two transposons in *B. fragilis*. The gene designated *tet(X)* acts by chemically modifying tetracycline in a reaction requiring oxygen. The resistance phenotype was expressed in *E. coli* grown under aerobic conditions. Tet(X) appears to be a soluble 44 kDa NADP-requiring oxidoreductase and is the only known tetracycline-modifying resistance determinant (131,132).

Chopra *et al.* have shown that among the known tetracyclines there is a division into two groups based on mechanism. Tetracyclines such as thiatetracycline and anhydrotetracycline have a bactericidal mechanism involving the inhibition of DNA and RNA biosynthesis, in addition to their inhibitory action on protein synthesis. Interestingly, these compounds show enhanced activity against tetracycline-resistant bacteria. These agents have no direct effect on the bacterial inner membrane, but drug exposure leads to release of cytoplasmic β-galactosidase (133-135). Recently, a new class of naturally occurring tetracycline molecules, the dactylocyclines, were reported to exhibit some activity against gram-positive tetracycline-resistant organisms bearing efflux determinants (136). These are the first naturally occurring tetracycline C2 amides which are not cross-resistant with tetracycline. Some interesting features of the molecular structure of these compounds are the inverted C6 stereochemistry: a β-oriented methyl group, and an α-oriented tertiary oxygen bearing a nitrogen-containing sugar. In addition, the C4a position bears a hydroxyl group, and a methoxy group has been incorporated at the C8 position (137).

Aminoglycosides - Although loss of permeability and ribosomal mutation (in the case of streptomycin) can lead to aminoglycoside resistance, drug modification is the primary mode of resistance found in clinical isolates. There now exists an extensive literature that documents the occurrence, activities, specificities and intracellular location of aminoglycoside-modifying enzymes (138). Among the recent developments in this area is the discovery of a bifunctional enzyme which has both N-acetyltransferase and phosphoryltransferase activity and confers resistance to all aminoglycosides except for streptomycin and neomycin (139). Aminoglycoside-modifying enzymes are typically plasmid-encoded (138) and frequently associated with transposons, which facilitates the wide dissemination of these resistance determinants. This is reflected in recent surveys using DNA hybridization techniques which suggest that typical aminoglycoside-resistant clinical isolates harbor more than one resistance gene (140).

High-level aminoglycoside resistance (MIC $\geq$ 2000 μg/ml) is now encountered in *Enterococcus* (64,141). Evidence suggests that the bifunctional enzyme is the most prevalent enterococcal aminoglycoside modifying-enzyme detected in U.S. isolates (139,142,143), which is likely due to the location of the structural gene on both a conjugative plasmid and a mobile genetic element (144). This is a disturbing trend since once a strain acquires high-level aminoglycoside resistance, it is no longer susceptible to the synergistic, bactericidal effects of β-lactam/aminoglycoside combinations (64) often used to treat serious gram-positive infections.

Mycobacterium tuberculosis - Recently there has been an alarming increase in the number of cases of multidrug-resistant *M. tuberculosis*, an infection which is often fatal (145-147). The AIDS epidemic and poor compliance on the part of those taking medication for *M. tuberculosis* have contributed to the problem (148,149). Clearly, one epidemic (AIDS) has set the stage for reemergence of another more contagious disease (tuberculosis) and in turn raised it to epidemic levels (150-152). For some time, it has been well established that combination therapy has been the rule for successful treatment of *M. tuberculosis* (153). Current drug therapy for *M. tuberculosis* has been extensively reviewed, but very little has been published about the mechanisms of resistance (154,155). The mechanism of isoniazid resistance was, however, recently elucidated (156). Clinical isolates that are resistant to isoniazid, a mycolic acid synthesis inhibitor, show reduced catalase activity. A single *M. tuberculosis* gene, *katG*, encoding both catalase and peroxidase, restored drug sensitivity to an isoniazid-resistant mutant of *M. smegmatis*. Deletion of this gene was associated with resistance in two clinical isolates of *M. tuberculosis*. One explanation

is that the active form of isoniazid is a metabolite requiring the action of the catalase-peroxidase enzyme (157,158). Recently a 59 kDa cell wall protein was isolated from *Mycobacterium chelonae* and was characterized as a porin. This unprecedented finding suggests that understanding heretofore unknown hydrophilic pathways may be useful in the design of new antimycobacterial agents (159).

Nitroimidazoles - Although resistance to nitroimidazoles in *Bacteroides* species is not common (160), several mechanisms have been reported (161). The mechanism which has received the most attention is that of reduced reductive activation of the nitro group, the first step in the bactericidal action of the nitroimidazoles. In the presence of metronidazole, resistant *B. fragilis* strains have been shown to exhibit lowered pyruvate:ferredoxin oxidoreductase enzyme activity compared to sensitive strains (162). It has been suggested that lactate dehydrogenase may compensate for this lowered enzyme activity, as lactate dehydrogenase activity is higher in resistant strains exposed to metronidazole (163). Metronidazole-resistant strains of the parasite *Trichomonas vaginalis* have recently been reported to exhibit reduced transcription of the ferredoxin gene, which, in similar fashion, would result in decreased activation of the nitroimidazole agent (164). In *Bacteroides*, transferable plasmid (165) and non-plasmid (166) mediated resistance has been reported. A plasmid from *B. fragilis* conferring low-level metronidazole resistance in *E. coli* was found to encode a protein of approximately 10 kDa (167), although the exact mechanism of resistance is still unclear. Whole cells or sonicated cell extracts of *E. faecalis* were found to completely inactivate metronidazole; this may be important in treatment of mixed infections (168).

The incidence of metronidazole resistance in *Helicobacter pylori* is significant, with 20-30% of isolates in North and South America, Europe and Australia exhibiting this phenotype (169). This can interfere with eradication of the organism, as metronidazole is a critical part of most therapy regimens, and resistance to this agent results in an increased percentage of treatment failures (170,171). Nitroimidazole resistance seems to be chromosomally mediated (169), to develop rapidly *in vitro* and *in vivo* (172) and remain stable for years (173). Although the mechanism of resistance in *H. pylori* has not yet been established, several hypotheses have been proposed. Due to the increasing susceptibility of resistant strains to metronidazole when cultured under anaerobic conditions, rather than in the more typical microaerophilic environment, it has been suggested that resistant strains do not normally achieve a sufficiently low reduction potential for the necessary nitro group reduction (174). Further speculation involves the possibility that nitroimidazoles may act through a different mechanism under microaerophilic conditions (161,175).

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Chapter 16. Proteases and Metastasis

Jack Henkin
Abbott Laboratories, Abbott Park, IL 60064

Introduction - The aggressiveness of cancer cells from primary tumors in colonizing secondary sites is the major cause of cancer mortality, and relies on a host of phenotypic properties including adhesion, chemotaxis, and angiogenesis. Critical is the ability of cells to enter and leave the circulation and to invade adjacent tissue. This requires degradation of extracellular matrix (ECM) via concerted action of proteases directed at various ECM components. Digestive activity evolves as a cascade in which inert proenzymes are successively activated by active enzymes preceding them in the chain. Inhibitors are also elaborated in tandem for control of the process, and protease/inhibitor balance appears to be abnormally regulated in malignant cells. This process has been reviewed (1-4), and while excellent correlative evidence has accumulated for a direct role of proteases in metastasis, it remains to be seen whether inhibitors of these enzymes will have a therapeutic impact. Newer results on the important proteinases are described.

Extracellular Matrix (ECM) - ECM includes basement membranes (BMs, basal lamina) and stroma. BM are thin tight membranes that separate immunologically distinct tissues. They surround blood and lymph vessels that tumor cells must pass through to metastasize, and consist of type IV collagen, heparin sulfate proteoglycans, laminin, entactin, fibronectin (FN), and other glycoproteins. Type IV collagen polymerizes in a sheet-like lattice connected to adjacent planes by S-S bonds. Laminin, with a cross-like shape, acts as a multifunctional connector. Separate domains bind type IV collagen, heparin sulfate, and cell surface receptors recognizing the YIGSR sequence of laminin. Entactin connects the short arm of laminin with FN and with the globular domain of type IV collagen. Destruction of lamina is the pathologist's sign of invasiveness and is the hallmark of malignant tumors (4). The underlying stroma is comprised of interstitial collagens, proteoglycans and glycoproteins, hyaluronic acid, elastin and FN (1). FN contains multiple binding domains for collagen, heparin and specific cell receptors having an RGDS sequence. Elastin is a hydrophobic protein forming proteinase-resistant elastic fibers through a network of crosslinks. Connective tissue cells residing in the stroma continuously degrade and synthesize the above components so that ECM turns over slowly but remains impermeable to cells and large proteins. Reconstituted ECM as used in *in vitro* matrix invasion studies often tend to be formed by coprecipitation of individually solubilized matrix proteins. These precipitates are weaker in tensile strength and more susceptible to proteolytic degradation than intact matrix, suggesting a structural role for the stromal cells beyond simply expressing the components.

In addition to forming a structural barrier, ECM also acts as a depot for biologically active molecules where they can be stored, and protected. Laminin itself contains a cell binding domain YIGSR sequence that binds to a receptor on metastatic cells and the peptide itself can inhibit metastasis (1). Another laminin sequence, SIKVAV appears to enhance tumor growth on its own via angiogenic stimulation (5). While not present in normal stroma, the stromal cells surrounding tumors express tetranectin (6), which can bind the kringle 4 domain of plasminogen (Pg). It is thought to anchor Pg to sulfated polysaccharides in the ECM. Vitronectin (VN), attached to ECM, binds PAI-1, the major inhibitor of Pg activators (PAs). PAI-1 is thus stabilized in an active form and distributed to resist PA-stimulated degradation. HT-1080 tumor cells adherent to FN synthesized 2-3 fold more urinary-type PA (PA) than cells adhering to VN, while adherence to the VN caused clustering of a uPA receptor to focalize its activity on the cell surface (7); thus, matrix is more than a passive bystander in the process of invasion.

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METALLOPROTEINASES AND THEIR INHIBITORS

Matrix metalloproteinases (MMPs) operate at neutral pH and are secreted by inflammatory and metastatic cells into ECM where they may degrade matrix protein. A number of distinct activities have been correlated with metastasis (1): type I collagenase (interstitial, MMP-1), type IV collagenase (72 kDa gelatinase A, MMP-2), 92-95 kDa gelatinase B (MMP 9), stromelysins 1, 2, 3 (MMPs-3, 10, and 11), and matrilysin (MMP-7). Sections of 29 esophageal carcinomas were stained for MMPs 1,2 and 3, and compared with normal epithelium. At least one MMP was detected in 59% of the marginal edges or deeply invasive portions of the tumors, with all 3 being present in 21%. Among tumors with invasion of more than 3 lymphatic vessels, only 25% had none of these MMPS. MMPs 2 and 3 were about twice as commonly secreted as MMP-1 (8). MMP function is highly regulated. Levels of mRNA for collagenase and stromelysin (ST) are increased by phorbol esters, PDGF and oncogenes H-*ras* and v-*mos;* all apparently through c-*fos* protein (9). Glucocorticoids and TNF-β negatively impact the mRNA transcription. All MMPs are secreted as inert proenzyme forms which can be activated, autolytically, sometimes by each other, or by plasmin (Pn). In all cases, an N-terminal fragment of about 80 amino acids is removed which carries a free cys-SH. The thiol is initially liganded to the essential metal of the enzyme, usually Zn^{+2} or Ca^{+2}, thus preventing the required interaction with substrates until peptide cleavage entropically favors its dissociation and replacement by water.

Gelatinases - The secretion of both gelatinases A and B has been frequently associated with increased metastatic potential (10). Their activation from proenzyme forms in fibroblasts enables degradation of type IV collagen films. While plasmin (Pn) can readily activate ST, and the ST can then stimulate gelatinase B, the activator for gelatinase A is unknown (11). The latter is only weakly detected by immunochemical staining in normal tissue or in benign growths or polyps, while most invasive carcinomas of the breast, stomach and colon are positive for this antigen, which attacks type IV BM collagen in a pepsin-resistant triple helical domain (12). Exposure of melanoma cell media to laminin induces 2-3 fold increases in the enzyme activity, and to degraded laminin yields a 6 fold increase (1). The C-terminal region contributes to the binding with inhibitors (TIMPs) and also is essential for activation of the proenzyme by an as yet unidentified plasma membrane activator (13). An immunologically similar enzyme (92kDa) has been associated with malignant transformation in rodent tumor cell lines. Human basal cell carcinoma has been shown to induce expression of the 92kDa protease in eosinophils within inflammatory infiltrates surrounding tumor nodules, thus recruiting protease from normal tissue (14). No such RNA was detected in circulating eosinophils or those found in Hodgkin's lymphoma.

The ability of human lymphoblastoid cells to invade an artificial matrix, or to grow and spread in a SCID mouse model has also been correlated with the expression of 72kDa type IV collagenase (15). Human serous ovarian tumors were examined with antibodies to this enzyme. Benign adenomas with well preserved BM, showed weak or no staining, while malignant ones with BM disruption showed strong staining in cells invading the stroma. Strongly staining cells showed no BM components in their surroundings, implying a role for the enzyme in invasive behavior (16). A 72 kDa protein in pericellular matrix could be cleaved by urokinase (UK) to a 62 kDa form. This has now been shown to be a precipitated form of type IV collagenase (17), and becomes gelatinolytic upon UK-catalyzed but Pn-independent cleavage, although it is not yet known if type IV collagen is a substrate. While this reaction was slow, tumor cells are known to often have very high levels of UK. A Pn catalysed processing of this type is known although contradictory results have been reported (17). Interestingly a rat glioma cell line secretes a type IV collagenase which can also be activated directly by human UK without involvement of Pn (18). In squamous cell carcinoma, especially of the oral mucosa, some interstitial (MMP-1) collagenase gene expression was detected in the tumor cells, but higher levels were observed in fibrous tissue immediately adjacent to the tumor (19); normal or hyperplastic mucosa were consistently negative.

Stromelysins - Stromelysins (ST1,2 and 3), secreted as 57kDa proenzymes, have broad specificity

for proteoglycans, laminin, elastin, FN and gelatin; with some activity towards type III IV and V collagens. Carcinogens cause activation of the H-*ras* oncogene in mice. First, papillomas appear, with only a few showing detectable ST transcript. Progression to carcinomas yields a majority with high levels of ST transcript (20). *Ras*-transformed rat embryo cells can metastasize after s.c. injection, while this is not true for cells cotransfected with adenovirus 2 E1a, although the latter remain tumorigenic. Only the former simultaneously expressed large amounts of STs 1 and 2 (21). In the same experiments, no correlation was seen with MMP-2 or MMP-9 (72 and 92 kDa); levels were very low, thus STs alone were effectively able to degrade type IV collagen *in vivo*.

Expression of the more recently discovered ST3 was examined at the advancing tumor edge in 107 head and neck squamous cell carcinomas (22). It was detected in 102 of these and in 17 of 21 metastatic nodes, while only 2 of 60 nearby normal tissues showed ST3 RNA (23). ST2 and type I collagenase are also overexpressed in this type of cancer, perhaps reflecting a synergistic action of these MMPs. Intensity of the ST3 response was much greater at the periphery of neoplastic islands, suggesting a significant invasiveness role, in agreement with the strong correlation of ST3 with cancerous infiltration. In both breast, and head and neck cancer ST3 is made mainly in fibroblasts immediately surrounding clusters of tumor cells, presumably under the control of a diffusible factor produced or induced by the cancer cells (23).

Matrilysin (MMP-7) - Smallest in the MMP family is matrilysin (MMP-7, PUMP-1). It is secreted as an inactive precursor of 28kDa and activated by N-terminal truncation to about 20kDa. It has been shown to degrade FN, gelatins, proteoglycans, laminin and entactin, the latter at several X-Leu or X-Ile bonds. Degradation rates were found to be 2-3 orders of magnitude higher than higher than with interstitial collagenase or 92 kDa gelatinase (24). MMP-7 has recently been shown to catalyze the hydrolysis of a specific Glu-Leu bond in prourokinase, thereby forming LMW UK and separating the catalytic domain from its receptor binding amino-terminal fragment (25).

The expression of MMP-7 RNA in samples of 8 out of 10 human gastric carcinomas and in 6 of 8 colon carcinomas was striking (26), while none showed expression of STs 1 or 2. None of these MMPs were expressed in nearby normal tissue, and MMP-7 production was demonstrated to be carried out by the tumor cells rather than in stromal cells. MMP-7 was found to be expressed by prostate tumor cells, and not by their surrounding stroma in 14 of 18 carcinomas (27). However, 3 of 11 normal prostates also showed transcript, tentatively ascribed to macrophages within inflamed local ductal epithelium. In these same tumors, 6 of 10 examined had type IV collagenase transcripts, while none of 4 normal tissues did. All sections positive for the collagenase were also positive for the MMP-7. Since MMP-7 can activate type IV collagenase, their tandem production might be of importance for invasiveness. When the MMP-7 gene was introduced into the tumorigenic but non-metastatic prostate cell line DU-145, transfected cells were much more invasive than "vector only" controls (28). The latter invaded the diaphragm of only 1 of 11 SCID mice after i.p. injection while the matrilysin-producing transfectants colonized 12 of 18 injected animals, indicating a role for MMP-7 in degrading basal lamina and stroma.

TIMPs - Tissue inhibitor of MMP (TIMP, TIMP-1) a glycoprotein of 28.5 kDa (peptide 20.7 kDa) stoichiometrically complexes with and inhibits all of the above MMPs. It inhibits artificial membrane invasion by tumor cells and reduces the number of metastases if coinjected with melanoma cells into mice. Its lowering via antisense TIMP RNA induced relatively indolent cells to display a metastatic phenotype (1). Elevated levels were found in tissue extracts from all of 31 human colon tumors, compared with their adjacent normal mucosa. In 23 tumors , the elevation was more than 3-fold and in 9 it was more than 6-fold (29). It was suggested that both TIMP and collagenase activity are coordinately upregulated in these tumors, with MMP activity probably dominating. Interestingly, exposure of human fibrosarcoma cells to TGF-β simultaneously induced both increases in type IV collagenase (latent 72 kDa and active 62 kDa) and an increase in TIMP, with the latter dominating to give a net reduction in invasive and collagenolytic activity (30). Invasion of Matrigel matrix by B16 melanoma cells could be reduced by 40-60% in cells

transfected with TIMP-1. These could be stimulated via a metallothionein promoter to 3-10 fold enhancement of TIMP message and further inhibition of invasiveness (31). An immunologically distinct inhibitor, TIMP-2, reacts with most MMPs in their active form but preferentially with the 72 kDa collagenase, and not with the 92 kDA enzyme. TIMP-2 can be isolated and purified as a reversible complex with the 72 kDA proenzyme and deletion mutations showed that the binding of TIMP-2 requires both the active site and the C-terminal tail of the gelatinase (32). The enzyme is essential for invasion of artificial matrix by human HT-1080 tumor cells reflected in the inhibitory effect of natural TIMP-2, which could abrogate the invasiveness without inhibiting chemotaxis. Recombinant TIMP-2 was able to inhibit degradation of smooth muscle cell matrix in coculture with human fibrosarcoma cells (33). TIMP-2 was transfected into rat embryo cells engineered so as to overexpress MMPs. These cells grow large nodules with muscle invasion when injected in flanks, and colonize the lungs of i.v. injected in nude mice. Clones expressing TIMP-2 formed much smaller nodules without muscle invasion. Only the highest expressing clone showed major reduction in the number of lung colonies (34). The epidermoid carcinoma, HEp-3 maintains metastatic ability in the chick chorioallantoic membrane (CAM) when passaged on CAMs but loses this behavior upon growth for 8 weeks in culture. While uPA, PAIs 1 and 2, both 72 kDa and 92 kDa type IV collagenases, and TIMPS 1 and 2 were measured over time, only an increase in TIMP-2 correlated well with the time course of the loss in metastasis (35).

While most of the above studies support the MMP/TIMP balance as a key factor, a recent study showed the need for cautious interpretation. It measured antigen for MMPs 2 and 9, and TIMPs 1 and 2 and their complexes in 8 lung tumor cell lines. The 2 most metastatic lines produced the most MMP-2, although TIMP-2 levels in these were even higher (36). Reverting to a more benign phenotype via transfection with K-*rev*-1 unexpectedly gave even more MMP-2 and less TIMP-2, and thus with this tumor-suppressing gene, other factors may dominate.

SERINE PROTEASES AND THEIR INHIBITORS

Secretion of a variety of serine proteases is characteristic of many tumor cells. In the case of Pg and PAs, the formation of Pn clearly provides a route for activating a cascade of pro-MMPs as well as direct attack on matrix components. Less well studied trypsin-like enzymes may contribute to the understanding of matrix degradation. Many cancer cell lines produce one or both isozymes of a tumor-associated trypsin-like enzyme (TATs 1 and 2), which are blocked by a specific inhibitor (TATI) first discovered in mucinous ovarian tumors. TATI has been shown to inhibit about 50% of the degradation of both the fibronectin and collagen of matrix by three tested tumor cell lines (37). However when degradation by one of these was enhanced by 7-8 fold upon addition of Pg, TATI could only reduce a small portion of the breakdown; a concerted action was inferred for Pn, TAT-2 and other proteases in ECM breakdown. TATs can also activate proUK. Of ten gastric carcinoma lines examined, two were found to be non-producers of MMPs, and these secreted four types of pro-serine protease (38). They included Pg-like, uPA-like and tissue kallikrein-like enzymes, as well as two trypsin-like enzymes of 24 and 26 kDa. The smaller of these when activated extensively degraded fibronectin, laminin and gelatins, and more slowly collagens.

Plasmin - Significant degradation by Pn of laminin and FN is well established (4), as is activation from their latent forms by Pn of MMPs, STs and elastase (39). Pn also attacks VN at limited sites (40), and, as VN stabilizes PAI-1, this may further accelerate generation of Pn and a proteolytic environment. Receptors for Pg/Pn with a preference for Pn have been reported on cells derived from mammary and colon carcinomas. Displacement of Pg from such sites showed that they were probably not fully saturated *in vivo* (41). Brief exposure of cells to Pn or other tryptic proteases leads to increased Pg binding capacity (42) and the catalytic efficiency of surface-bound Pn may be up to 6-fold greater than the free enzyme. This has been associated with the formation of C-terminal lysine residues and in one case particularly with the cell surface α-enolase (43), possibly the primary Pg receptor. Notably, the rates of quenching of receptor-associated Pn by

α_2-antiplasmin and α_2-macroglobulin are much slower than that of free plasmin (44).

Urinary Plasminogen Activator - Urinary-type plasminogen activator (uPA) is secreted as a proenzyme (proUK) of 54 kDa and can bind to a specific glycolipid-anchored cell receptor (uPAR)(45). uPA can also be found in a LMW form (32 kDa) which does not bind this receptor, and recent results have shown that the LMW form may be formed by MMP-7 (25). UK or Pn may also slowly cleave and release the uPA-UPAR complex (46). Active UK is generated upon cleavage of the Lys 158-lLel59 bond of proUK, a reaction catalyzed by Pn, kallikrein, CATs B and L and TATs (4). Increased levels of uPA and/or of uPAR have been highly correlated with metastatic and invasive behavior in a wide variety of tumor cells and these are thought to play a pivotal role in generation of the ECM degrading protease cascade (47-49). In addition to its presumed main mechanism of activating Pg, UK is also known to directly attack FN, yielding 4 polypeptides. FN thus cleaved is more rapidly and completely degraded by either Pn or Cat D (50).

The level of uPA was 2 to 3-fold higher in 42 gastric carcinomas than in adjacent normal mucosa (51). Of six human melanoma cell lines tested for spontaneous lung metastasis after s.c. injection into nude mice, only the two lines producing uPA gave a high rate of colonization (52). PAI-1 was also made by these two lines and may be required for fine tuning of Pg activation. When human rectal carcinoma cells were injected into nude mice and metastatic foci were repeatedly collected and reinjected into new animals, it was possible to isolate a highly metastatic subline with a higher uPA content than the parental line (53). The few colonies arising from the injection of thousands of osteosarcoma cells also showed substantial enrichment of uPA when reisolated (54). Biopsy specimens of gastric and esophageal carcinomas were compared with adjacent normal tissue, and 38 of 39 pairs showed considerably greater uPA antigen in the tumor tissue, although there was heterogeneity between patients. By comparison tPA was unchanged, and uPA was particularly elevated with ulcerating carcinomas (55). Tumor levels of uPA and of PAI-1, but not tPA have been shown to be of significant prognostic value in breast cancer independent of other markers (56). The uPA may indicate invasiveness while the PAI may also reflect a high degree of neovascularization; or PAI-1 may represent an ongoing defense against the tumor invasion (57). It is not yet established whether the tumor cells themselves make uPA or induce it in surrounding stromal cells or macrophages. Antigen measurements on cytosols from 130 breast tumors showed about 5-fold increases in uPA on average comparing malignant and benign growths. However, in the same study PAI-1 and PAI-2 were reported to increase about 70 and 30-fold, respectively (58). The amount of uPA in tumor extracts is correlated with the grade and extent of bladder cancer, and the survival rate in the latter was much poorer in patients whose uPA content in the tumor was above the median value (59). Likewise, the tumor level of uPA in 20 patients with invasive cervical cancer of the uterus was a strong predictor of pelvic lymph node metastasis (60). Increased levels by 2-3 fold of circulating plasma uPA were found in patients with acute nonlymphoblastic leukemia and the uPA level correlated with mortality (61).

The uPAR, which has now been cloned, controls and focuses the invasive potential of uPA (45). Immunohistochemical staining of colon tumors showed that tumor infiltrates had 10-fold higher uPA levels, but that the uPA was mainly produced by stromal fibroblasts (62). Since colon tumor cells are rich in uPAR, the cancer cells induce uPA production in their surrounding stroma for recruitment to their receptors (62). Cell matrix was hardly degraded by the colon carcinoma HT29, even though this line contains 100,000 uPAR per cell (63). However matrix invasion was greatly enhanced when the cell was transfected with the uPA gene. Radiolabled A549 or Calu-1 lung carcinoma cells became more invasive of human amnion if induced by cytokines to display a greater level of surface uPA binding capacity (64). Anti uPA or PAI-2 could abrogate this, defining a major role for uPAR-bound UK; and PAI-1 inhibition of cell invasion could be removed by blocking the interaction of PAI-1 with matrix vitronectin. It has been possible to distinguish 3 varieties of non-small cell lung cancer with respect to uPA and uPAR. Lines of squamous cell origin did not produce or bind remarkable amounts of uPA, while large cell carcinoma lines both produced high levels of the enzyme and or receptor (65). Mesothelioma lines did not express the

enzyme but were able to bind significanty quantities. A comparison of 6 benign and 10 malignant epithelial ovarian tumors showed the latter to have >2 fold higher levels of uPAR (66).

Mixed feeding experiments between uPAR-producing cells lacking their own uPA, in coculture with cells that make uPA but not receptor, led to enhanced degradation of smooth muscle cell matrix (67). Similarly, a mixture of mouse cells transfected with the human uPA gene and ones transfected with the uPAR were more efficient by 3-fold at CAM penetration than either of the cells alone (68). A study of CAM invasion by various carcinoma lines found that all invasive ones had surface-bound uPA, but this was not sufficient to allow penetration since some non-invasive cells had uPA/uPAR (69). Only the lines also producing interstitial collagenase were efficient at invasion and loss of this enzyme on passage correlated with the loss of invasiveness. The invasiveness of 2 melanoma lines that bind Pg to their surfaces was compared in a variety of artificial matrix systems. The cell with uPA was much more effective at Pg activation compared with that having tPA, and also at matrix invasion which could be blocked by added aprotinin or by prior removal of the Pg (70). Both interstitial and BM type matrix degradation by the human melanoma M24met line was demonstrated to be dependent on both uPA and MMP activity as shown by inhibition by either PAI-2 or TIMP-2 (71). The temporal effects of these inhibitors on different types of matrix breakdown suggested that collagen digestion was protected against by matrix glycoprotein. UK/plasmin activity was not required for MMP zymogen activation but was essential to degrade these in advance of the MMP attack on collagen. Interestingly, the non-catalytic domain of uPA has been shown to have growth factor activity (72,73).

The specific blocking of UK has been shown to inhibit dissemination or metastasis in animal models. Nude mice receiving s.c. human squamous carcinoma HEp3 developed tumors with high uPA level which spread to lung and lymph nodes (74). Introduction of anti-uPA to the circulation in this study prevented muscle invasion, but not metastatic spread. When non-tumorigenic human HOS osteosarcoma cells were transfected with oncogenes, a variety of sublines were generated whose spread to mouse lung after i.v. injection closely correlated with cell-associated uPA activity, as mitigated by PAI-1 (54). Pretreatment of the most aggressive line with anti-uPA led to a 7-fold reduction in the number of tumors. Displacement of uPA from uPAR in prostate tumor cells by means of a uPAR-binding peptide reduced invasion by about 80%, and aprotinin, anti-uPA and PAI-2 all significantly inhibited invasion (75). Similar results were also recently observed with an ovarian tumor cell (76). Suramin has been recently reported to displace uPA from uPAR at concentrations between 10^{-5} and 10^{-4} M, and this was speculated to account for some of the anti-invasive properties of this drug (77).

CATHEPSINS AND THEIR INHIBITORS

Cysteine Cathepsins - Two cysteine proteinases (CPs), cathepsins (Cats) B and L, have been correlated with malignant transformation. Cat B is secreted as a precursor form of 40kDa and exists either as a single chain of 30kDa or two linked chains of 25 and 5kDa. It is an endopeptidase of broad specificity, with acid pH optimum, but can continue to function at neutral pH in the presence of large protein substrates (78). Laminin, FN and type IV collagen are all degraded by it at both acidic and neutral pH. Evidence continues to accumulate suggesting a role for Cat B in tumor malignancy, but the distinction between more and less malignant tumors is more one of localization and regulation by cysteine proteinase inhibitors (CPIs) than absolute amount (78). While still mainly found in lysosomes, the enzyme is 4-30 fold more abundant in the plasma membrane fraction of tumor cells than in those from normal tissue. Membrane-associated enzyme is relatively resistant to inactivation by CPIs and by neutral pH. Early action by it may create an acid local microenvironment, further enhancing activity at the cell interface with basement membrane. The aggressiveness of bladder tumors and their ability to degrade laminin has been correlated with the amount of plasma membrane-associated Cat B . When invasive EJ and non-invasive RT4 transitional cell carcinomas were implanted into nude mouse bladders it was found that the EJ cells had mature Cat B diffusely spread through the cytoplasm while it was localized to

lysosomes in the RT4 line. The EJ cell Cat B was mature while the RT4 mainly was in precursor form. Laminin degrading activity was unique to the plasma membrane of the invasive cells and the degradation products were those expected from pure Cat B treatment (79).

Cat L was first recognized as the MEP (major excreted protein) found in transformed mouse fibroblasts. This was discovered to be a 39kDa precursor of the smaller lysosomal enzyme. Cat D may be involved in its processing. In spite of being tagged for lysosomal uptake with mannose phoshate, the bulk of overproduced Cat L from transformed cells is secreted. Thus, this acid cysteine protease is uniquely set for export into the lysosomes of neighboring, non malignant cells. This has been shown to interfere with the surface presentation of antigens, and may mitigate the immune response to tumor cells (80). *In vitro* Cat L is the most potent lysosomal protease in degradation of elastin, laminin and collagen and it appears to accommodate very hydrophobic residues next to substrate cleavage sites better than Cat B. The invasiveness through artificial matrix of ovarian cancer cells (HOC-I), which display both proUK and Cat B on their surface, through artificial matrix was >80% inhibited by neutralizing Ab against UK, but not by anti-Cat B (81). The fact that >70% inhibition was achieved by E-64, a synthetic inhibitor of both Cats B and L, suggests that Cat L may be important in this case, possibly by activating proUK. The secreted proenzyme is stable to neutral pH but shows little or no activity except at acid pH. A notable exception is the observation that Cat L can activate proUK into UK reasonably efficiently at neutral pH (82). At low pH, the protease goes on to quickly destroy the elicited UK activity. Thus Cat L may trigger UK activation in some tumor cells. Cat B has also been shown capable of converting free or receptor-bound prourokinase into its active form (83). Thus Cat B or Cat L may initiate a cascade ultimately forming plasmin and active collagenases. The main intracellular inhibitors are the stephins (11kDa) members of the cystatin superfamily. These are reduced in tumors and are poor at inhibiting cathepsin B compared to Cat L (78). In murine models, highly metastatic subpopulations isolated after lung colonization, showed higher proportions of Cat B in their membrane fractions and much lower amounts of membrane associated CPIs (80). CPIs are generally very stable but they can be inactivated by Cat D. Establishment of the role of Cat B in extravasation has been hampered by the lack of specific inhibitors.

While Cat L overexpression can be induced in mouse cells by transformation, growth factors and tumor promoters, its importance in human malignancy was questioned. Recently however, an examination of 100 human tumors ranging over 14 organs, has shown that the majority have substantially increased levels of cathepsin L RNA compared with the corresponding normal tissue, particularly for kidney, testis and lung (84). One study examined Cat B and Cat L activities as well as CPI activity in ductal breast cancer matched with normal tissue controls from the same patient (85). There was no correlation of these with lymph node metastases but very elevated Cat L and somewhat elevated Cat B, along with diminished cystatins were associated with poorly differentiated carcinomas. Extracellular cystatins (13kDa) and intracellular stephins, the latter found in acidic (A) and neutral (B) forms, are the main inhibitors (CPIs) of the cysteine proteinases. In rat embryo fibroblasts, closely related cell lines were examined having either transformed, or tumorigenic, or ras-transfected metastatic phenotypes. The latter showed only a slight increase in Cat L and no increase in Cat B. However they had 4-15 fold lower levels of CPIs and thus much less regulation of these cathepsins (86). Very similar results were observed in *ras*-transformed mouse 3T3 cells (87).

Cathepsin D - Cathepsin D (Cat D) is an aspartyl proteinase having optimal activity and fairly broad specificity at low pH. It is located mainly in lysosomes but can sometimes be associated with membranes, and has no known natural inhibitor. Most work on Cat D's role in tumor invasion and metastasis has been carried out in breast tumor cells. Cat D can activate Cats B and L and can also act as an autocrine mitogen, possibly by activating latent forms of growth factors (88). Its transfection into a mouse tumor cell greatly increased metastatic ability (89). High levels of Cat D in breast tumor cytosols is well correlated with poor prognosis and is associated with aneuploidy and amplification of the c-*myc* oncogene (90). Prediction of poor survival by Cat D level was

independent of receptor status, lymph node invasion or age. Recent studies also indicate a correlation between Cat D level and prognosis in endometrial cancer (91), and in head and neck cancer (92). Cat D is found in some malignant melanomas but never in benign nevi and it is also present in the ascitic fluid of patients with ovarian cancer. A Cat D inhibitor, pepstatin, albeit not specific, delayed ascites formation in tumor bearing mice. Nine clones of MCF-7 and six other lines of breast cancer cells were compared for their ability to invade through Matrigel and to secrete Cat D (93). No correlation was found, and pepstatin did not inhibit invasion. It appears that Cat D is probably not causal in invasiveness but rather is formed by lymphocytic cells infiltrating the tumor, whose presence is itself a prognostic of poor outcome.

Regulation and potential therapy - Invasiveness related to protease/inhibitor balance appears controlled by autocrine and paracrine factors coupled with a cell's differentiation state. An aggressive mouse mammary tumor line could enhance ST (transin) and uPA production and diminish TIMP in an indolent related line via heat labile factors from its conditioned media (94); effects could be simulated by basic FGF and EGF. TGF-β, TGF-α and EGF modulate PAI and PAs in human lung carcinoma lines (95). Cytokines and chemotactic factors can be used to recruit monocytes to infiltrate and supply proteases (96). IL-1B induced PA increases in a human astrocytoma line, and TGF-β and IL-1β modulated PA activity in osteosarcoma cells (97). Thus cytokines and growth factors may be used to mitigate metastasis via their effects on proteolysis.

Synthetic inhibitors of specific proteases may be therapeutically useful if the target enzyme is functional in metastasis. E-64, an inhibitor of Cat B, appears non-toxic and blocks mouse lung colonization by injected bladder tumor EJ cells (98). BCG binds to FN and protects it from digestion by Cat B; this prevents motility peptide formation and blocks membrane invasion by EJ cells (99). Nafamostat (FUT-175), a serine protease inhibitor prevents liver metastasis in mice receiving colon carcinoma cells via the portal vein (100). Tetracyclines have anticollagenase activity via metal ion binding and their testing against metastasis has been suggested (101). Suramin strongly blocks melanoma and mammary tumor invasiveness and it may function by inhibition of heparanase (102), Cat D secretion (103) and/or uPAR (77). A specific high affinity inhibitor has also been described for matrilysin (104) and such compounds are being actively sought against other MMPs. Weakly binding lead compounds with some specificity for UK have been reported (105).

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Chapter 17. Recent Developments in Cell Based Mechanism Screens

Stephen J. Gracheck, Paul F. Miller and James S. Marks
Parke-Davis Pharmaceutical Research
Division of Warner-Lambert Company
Ann Arbor, MI 48105

Introduction - Screening to identify new chemical entities for potential use as animal or human therapeutics has evolved to a highly specific applied science. The merging of molecular biological sciences and computerized robotics has led to an unprecedented ability to quickly screen large chemical or natural product libraries against specific molecular targets. Not only is this task accomplished rapidly, but the responses generated can provide detailed information as to the specificity for molecular targets to evoke a cellular response. The recent decade has seen a tremendous growth in receptor binding assays for screening many therapeutic targets at the molecular level. However, *in vitro* binding assays utilizing either immobilized or soluble receptors, cannot assess the cellular response effected by an agonist or antagonist binding to a receptor. The resulting cellular response may or may not be determined from the *in vitro* event, and in some cases traditional pharmacological methodology then must be used to assess cellular effects. Additionally, any protein conformational alterations resulting from reconstitution of the receptor may mask a potential interaction of a compound. These shortcomings can be overcome by using whole cell based screening systems.

Traditional whole cell based screens use isolated tissues which naturally express a receptor of interest. Many current methodologies use cell lines constructed to express a specific receptor. In this case, the receptor of interest is cloned and expressed in compatible cells and is presumably presented in a correct conformation. The advantage to using cell based constructs is the ability to determine secondary effects such as signal transduction modulation. The change in activity of a coupled signal or target pathway can be detected by several means including the use of gene fusions. The reporter fusions can feature a biochemically distinguishing phenotype to assess the effect of a molecule binding to a target receptor. A potential disadvantage in using cell based screens is the inability to distinguish receptor binders which are toxic to the cell at relatively low concentrations. This chapter is directed towards reviewing recent developments in cell based, high volume mechanism screening for the discovery of potential human therapeutics. Some of the assay systems described have yet to be reported for drug screening purposes, however the methodology may be adaptable to such an application when warranted.

Adhesion - Activation of the expression of neutrophil adhesion molecules at the cell surface has been widely used as a cellular endpoint for a multitude of biochemical modulatory events. Both protein kinase C and calcium-calmodulin activity have been studied by means of neutrophil adhesion assays (1). This high volume 96-well screening procedure used as an endpoint stimulus specific adhesion as measured by the ratio of neutrophils adhering versus those remaining in the supernatant when comparing stimulated to basal adhesion. Adhesion was assessed by measuring the release of ^{51} chromium from lysed cells in the supernatant of test versus control wells. This assay methodology successfully detected protein kinase C inhibitors, staurosporine, H-7, and HA 1004 by their blocking of neutrophil adhesion following stimulation. Additionally, compounds which modulate protein kinase C activity were also shown to be active in this assay. Assays such as this could be used to evaluate other adhesion molecules such as VCAM or ICAM either directly via interaction with their respective receptors or indirectly through modulation of protein kinase C, cytokines, or other growth factors (2-4). Cell based assay systems have been described for studying the modulation of ICAM-1 but have yet to be reported for use in screening strategies (5-7).

Cytokines - Interleukins are a family of polypeptide hormones which are involved in a multitude of inflammatory, immunologic and neurological events. *In vitro* screening of cytokines IL-1, IL-2, IL-4 and IL-6, and their respective receptors by binding assays have been widely reported. Cell based screening of IL-1 and its receptor has been described which utilize assays with varying degrees of complexity. The

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original assay employed oil centrifugation methodology which was not suitable for mass screening (8). Later variations including a multistep plate filtration assay and 96-well high volume format have been described (9). These differ in the relative degree of complexity and time involved while providing equivalent binding interaction data. The development of the 96-well format has permitted the implementation of rapid screening while generating comparable binding kinetics as compared to earlier screening methodologies (10, 11). This assay, typical of others for cytokine receptors, employed Raji, EL-4 or 70Z/3 cells naturally expressing the IL-1 receptor. The protocol involved mixing ^{125}I-IL-1 with IL-1 expressing cells, allowing for binding, followed by washing and filtering off the unbound radioligand. The selection of a ligand binding filtration medium with low background was found to be crucial to the relative precision and reproduciblity of this assay. This type of screening is not compromised by low affinity of the ligand for its receptor, with K_D values between 10pM to 2nM (10, 12). Ligand-receptor interactions with relatively low K_D values will be subject to loss of precision due to dissociation during the wash and filtration steps of similar protocols.

Platelet activating factor (PAF), a phospholipid, has potent modulatory effects in both the immune system as well as several major organ systems, including the cardiovascular system. PAF arises from stimulated immune cells, notably neutrophils, mononuclear phagocytes, basophils, platelets and endothelial cells. Assays to identify modulators of PAF have been described using a number of specific cell based systems (13). These assays identified several types of PAF antagonists, including PAF analogues, CV-3988 and Ro 10 -3704, PAF inhibitor RP 48740, BN 52021 and kadsurenone. A 96-well screen using human neutrophils was subsequently described which searched for PAF antagonists. This assay utilized neutrophils and tested for inhibition of elastase release upon stimulation with PAF. The above referenced compounds were shown to perform generally equivalent to the results obtained from the original screening methodology. PAF also was shown to bind to a neuronal cellular PAF receptors which were coupled to a signal transduction pathway in NCB-20 cells, a hybrid cell line of mouse neuroblastoma and Chinese hamster embryonic brain cells. Binding of PAF caused a rapid formation of phosphatidylinositol coupled to intracellular calcium mobilization (14). PAF antagonist, BN 50739 inhibited these coupled effects as did a protein kinase C activating phorbol ester. PAF also stimulated GTP binding to a subset of G-proteins which also was inhibited by BN 50739. The authors suggested that PAF receptors in these cells are linked via a G-protein secondary messenger system to phoshatidylinositol levels modulated by phospholipase C, and also control intracellular free calcium levels. While such a method has not been reported for screening of PAF agonists or antagonists, it is clear that cell based screening using such a system would provide not only competitive binding data but also coupled effects on G-protein linked pathways.

Endothelins have been the focus of many cell based screening strategies. A high volume 96-well cell based system using an embryonic derived aorta clone which expressed high-affinity ET-1 receptors was recently described (15). The screen used a microtiter vacuum filtration plate with PVDF filters to separate bound versus free ^{125}I-ET-1. Typical monophasic saturation kinetics were observed and identified ET-1 binding peptides which produced K_i values consistent with earlier reports. A similar methodology using bovine cerebellar membranes containing ET receptors, fixed to a 96-well plate, was the basis for a competitive binding assay also using ^{125}I-ET-1. This screen demonstrated less non-specific binding and higher specificity as compared to filtration methodology and allowed for the quantitation of the conversion of big ET-1 to ET-1 (16). Since endothelins are now being found in other tissues, in particular the central nervous system, research examining the function and secondary messenger system of specific tissue endothelin receptors is being extensively pursued. One such study characterized endothelin receptor subtype binding and the coupled signal transduction pathway in NG108-15 cells, a hybrid mouse neuroblastoma-rat glioma cell line. The results indicated the abundant presence of endothelin-1 receptors, including both low and high affinity receptors, which bound ^{125}I-endothelin-1 and demonstrated competitive kinetics with endothelin analogues (17). Additionally, endothelin binding was shown to be coupled to phosphoinositide turnover and this effect could be modulated by protein kinase C. A cell based screen utilizing such a system has yet to be reported, however this application, as opposed to *in vitro* receptor binding screening, could provide both competitive binding and coupled secondary messenger responses.

Growth Factors - Growth factors are proteins which modulate cell proliferative and differential responses by effecting signal transduction pathways. Cell based screens to detect antagonists and agonists have been reported for fibroblast growth factor, epidermal growth factor, transforming growth factor, and insulin-like growth factor. The majority of the microtiter based screens were developed to study the effects of cellular effector binding to cognate membrane receptors to study the resulting biochemical or physiological

response. As the secondary messengers became known for many growth factors, many may become suitable targets for screening of agonists or antagonists for therapeutic efficacy.

Fibroblast growth factors (FGFs), also known as heparin-binding growth factors, comprise a group of five polypeptides which target plasma membrane heparin binding receptors and effect a wide variety of physiologic and pathologic responses (18). A recent description of a cell based microtiter assay system to evaluate interactions between basic FGF and heparin-like molecules provides a means for rapidly screening compounds for modulatory activity (19). The assay tests for the interaction of compounds that inhibit the binding of RO-12 UC cells expressing syndecan, a heparin sulfate proteoglycan, to prebound basic FGF in 96-well microtiter plates. The binding was shown to be competitive with heparin and heparin sulfate but not other sulfonated cellular factors or modified heparin like compounds. The assay endpoint utilized the incorporation of $^{35}SO_4$ into heparin sulfate recovered from cell surface membrane preparations. Oligosaccharides which were previously reported to inhibit in *in vitro* receptor binding assays also were shown to be effective in reducing bound heparin in the 96-well format.

Transforming growth factor-beta (TGF-beta) comprises a group of polypeptide growth factors with a multitude of physiologic effects. Two recent reports of 96-well cell based assays examined the effects of TGF-beta 1 on plasminogen activator expression from bovine aortic cells, and on its effect as an inhibitor of hematopoietic cells. The effect of TGF-1 on plasminogen activator expression in a 96-well format was shown to be dependent on several controllable variables and exhibited a bifunctional response of either induction or inhibition of plasminogen activator expression and also may be used to evaluate angiogenesis (20). A 96-well cell based assay that tests for the effect of recombinant human TGF-beta 1 on macrophage proliferation has been described and detects binding of TGF-β-1 to receptors which selectively regulate proliferation (21).

Epidermal growth factor (EGF) is a ubiquitous peptide implicated in several pathological situations, most notably neoplastic diseases where there is an overabundance of EGF receptors. Several 96-well cell based assay systems suitable for high volume screening have been described. One system utilized a cloned human EGF receptor gene transfected into 3T3 cells. The assay tested for EGF stimulated mitogenesis as an endpoint. This screen was able to detect competitive binding to the EGF receptor as indicated by a loss of EGF induced mitogenesis (22). Subsequently, a more rapid and sensitive assay was developed using biotinylated EGF. This assay was kinetically characterized and shown to be similar to the native ligand-receptor interaction in its ability to detect antagonists or agonists (23, 24).

Insulin-like growth factor (IGF) bioactivity assessment was developed for two cell based systems. These assays used mitogenic or metabolic activity as an endpoint for IGF modulation of 3T3 fibroblasts (25). IGF-I, IGF-II and insulin all demonstrated modulatory effects in both assay systems while epidermal growth factor was inactive. Both assays were able to detect differences among IGF analogs in modulating either mitogen or metabolic activity.

ATP dependent potassium channel is involved in a variety of physiological responses including insulin secretion, blood pressure regulation and neuronal substances. A 96-well cell based screen for detecting potassium channel modulators was developed which utilized adherent clonal human medulloblastoma TE671 cells and measured the efflux of ^{86} rubidium as an endpoint (26). The screen was validated using diazoxide and calcitonin gene related peptide (CGRP), known activators of the K_{ATP} channel, as well as glibenclamide as an inhibitor of the same channel. However, limitations of the assay include the inability to distinguish cellular toxins, oxidative phosphorylation uncouplers, and importantly, any activity associated with modulation of voltage-dependent K-channels.

Dopamine receptors are part of the superfamily of G-protein-coupled receptors, and *in vitro* binding assays have been used extensively in screening programs to identify potential neuroleptic compounds. The D_1 and D_2 dopamine receptors originally were thought to be the predominant species found in the brain and were widely used in screening programs to identify agonists or antagonists. Recently distinct D_3 , D_4 and D_5 dopamine receptor subtypes were identified and cloned, and the D_3 receptor was successfully expressed in CHO cells (27). The D_3 receptor exhibited differential dopamine binding when compared to the D_2 receptor in CHO cells and was unresponsive to guanylnucleotides indicating a potentially different G protein coupling. Dopamine agonists bound both receptors equally, while autoreceptor-selective agents bound the D_3 receptor with greater avidity. The ability to differentiate binding to dopamine receptor subtypes may

allow for selected screens of targeted receptors. While no reports of cell based screening using cloned dopamine receptors have been reported it appears that the methodology exists to institute such screening.

Novel applications of receptor binding assays can be thought of in terms of cell-based screening systems when the receptor of interest is localized to a vesicle. An example of this is the recent report of G-protein-coupled adenosine A_1 receptors in coated vesicles of the mammalian brain (28). This system showed agonist/antagonist affinities and kinetic data comparable to standard radioligand binding experiments using membrane fractions from brain.

A high throughput, rapid cell based screen for detecting G-protein coupled receptor modulation was recently described (29). A frog melanophore based screen identified modulators which are functionally active for β_1-like adrenergic receptors coupled to guanine nucleotide stimulatory proteins. The 96 well format utilized recombinant melanophore pigment dispersion as an endpoint, controlled by intracellular cAMP, and was detected by changes in color and light transmission. This assay was shown to detect both agonists and antagonists of G_s-protein coupled adrenergic receptors, specifically the β_2 adrenergic receptor, which modulate intracellular concentrations of cAMP. A similar screen also was developed to identify modulators of G-protein coupled receptors linked to phospholipase C. Both agonists and antagonists for murine bombesin receptor, expressed in melanophores, were shown to modulate pigment dispersion in a dose responsive manner and were directly related to the protein kinase C activation state (30).

Cell based screening systems for identifying novel antiinfective agents have long been practiced. However, the methodology utilized for many years relied on a simple growth inhibition endpoint with no information as to the mechanism of action. Many current antibacterial screens utilize gene fusions with reporter mechanisms in targeted pathways of interest. Generally, the fusion is placed under the control of a particular promoter to evaluate the transcription of a gene of interest. Transcriptional activation or repression is then assessed by means of the fusion protein generally acting on a chromophoric substrate. Use of this technology for high volume screening has been reported for a number of antibacterial targets. Examples of this include *lacZ* fusions producing a fusion-β-galactosidase protein under the control of tetracycline resistance promoter of *E. coli*, the erythromycin induced promoter of *ermC*, the chloramphenicol resistance gene promoter of *S. aureus*, and the use of *lacZ* fusions in SOS repair regulated proteins to detect DNA biosynthesis inhibitors (31, 32). For antibacterial targets where the genetic basis for resistance is either unknown or difficult to study from a regulatory standpoint, cell based screening systems have been developed which are targeted but still semi-empirical in nature. An example of this is the development of a screen for detecting vancomycin-like inhibitors of cell wall biosynthesis from natural product sources (33). Two screens were described, one based on relative sensitivity for unknown compounds to inhibit vancomycin resistant and sensitive bacterial isolates, and the second which was based on a competitive binding differential. The second approach screened for unknown compounds which competed for an active site occupied by a synthetic analog, diacetyl-L-lysyl-D-alanyl-D-alanine. This latter screen was shown to be much more selective in identifying novel glycopeptides with vancomycin-like binding. Additionally, antiviral screening using virus specific enzymes or viral transcription factors expressed in mammalian cells has been widely reported (34, 35).

The potential for screening mammalian receptors in unicellular organisms has generally received little attention. There is a bias that mammalian receptors or enzymes are not posttranslationally modified in true fidelity to their natural environment and cannot predict coupled receptor binding events, e.g., signal transduction. However, the ease with which unicellular organisms are propagated and manipulated in high volume screening makes a strong case for their application.

For example, the *E. coli* aspartate receptor transmembrane region was fused to the cytosolic portion of the human insulin receptor containing a phosphotransferase activity and expressed in *E. coli*. The resulting fusion protein was active as a kinase and was stimulated upon binding of aspartate although the phosphorylation substrates were different than that of the intact insulin receptor (36). Additionally, the expression of β_1 or β_2-adrenoreceptors in *E. coli* and their use in high volume screening produced similar or identical binding kinetics when compared to identical receptors screened *in situ* from isolated membrane preparations (37, 38). A note of caution in the use of bacteria to screen cloned mammalian receptors is the presence of endogenous bacterial receptors which can compete for agonists or antagonists of the targeted receptor. An example of this is the binding of common neurotransmitters by *E. coli* and *P. fluorescens* which may lead to false or ambiguous screening results (39, 40).

The use of yeast to screen for coupled receptor-effectors appears to be a viable alternative to mammalian cell screens. A *Saccharomyces* screen expressing functionally and kinetically equivalent β_2 adrenoreceptors was shown to exhibit a signal transduction coupled response mediated by a yeast GTP binding protein of the pheromone response pathway (41) Similarly in yeast, the coupling of adenyl cyclase activity to G protein phosphorylation via a RAS like function has been described and could provide the basis for screening modulators of adenyl cyclase or inhibitors of G protein signaling (42).

Intracellular receptors as targets for pharmacologic intervention have received increasing attention. The intracellular targets for steroid and non-peptide hormones were identified at the molecular level as acting as transcription control elements, and a considerable number have been cloned and expressed. A striking feature of these results is the conservation of receptor amino acid sequence and structure which is independent of ligand diversity, leading to the referral of these as an intracellular receptor superfamily (43). Until recently, the screening of intracellular receptors was not practical due to a lack of suitable detection methods for identifying modulation of an intracellular receptor and its ligand. Application of a screening method called the cis-trans assay appears to be amenable to high volume screening of these targets (44). The cell based screen uses two response elements which are transfected into mammalian cells. The interaction of a constitutively produced target intracellular receptor an associated promoter is the basis for this screen which requires the binding of the intracellular target receptor complexed with its activating ligand. Following complexation and subsequent binding to the associated promoter, transcription of a reporter gene ensues and a biochemical readout is observed as a measurement of receptor-ligand interaction. This technique for screening for modulators of gene transcription reportedly offers the ability to perform high volume cell based screening and opens up the ability to screen for modulators of hormonal controlled responses (45).

A recent development in receptor screening, whether *in vitro* or cell based, is the patenting of the receptor or cell line for use as a screening tool. Examples of recently filed cell based assays include screening for antagonists or agonists of: interferon gama receptor (46); oncoprotein modulation (47); phospholipase A_2 (48); A_1 adenosine receptor (49); human granulocyte macrophage colony stimulating factor (50); MAP2-kinase (51); c-myc (52); retinoid receptor (53); various HIV-1 and viral targets (54); the serotonin 5HT1c receptor (55); the muscarinic acetylcholine receptor (56); and transcriptional control of growth factor expression (57, 58). This short list is indicative of the ongoing activity in developing specific and selective screens for the discovery of novel therapeutics using high capacity cell based, methodology.

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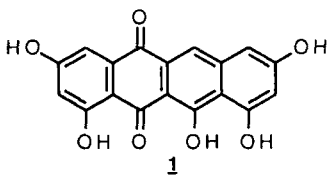
Chapter 18. Antineoplastic Agents

Robert P. Hertzberg and Randall K. Johnson
SmithKline Beecham Pharmaceuticals
King of Prussia, Pennsylvania 19406

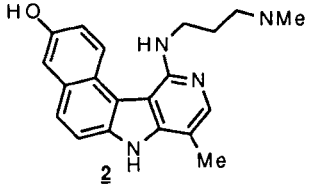
Introduction – The discovery of new compounds for the treatment of cancer continues to be the subject of intensive effort. A common strategy of antineoplastic drug discovery in the past several years has been to discover the way by which an effective anticancer agent works and use this knowledge in a mechanism-based drug discovery program to find new structures that work by a similar mechanism but have greater activity and/or fewer side-effects. This has been accomplished both through natural product screening and chemical synthesis. As a result, several new agents have entered clinical trial, including inhibitors of topoisomerase I and II, antimitotic agents, inhibitors of thymidylate synthase, and agents that bind to DNA covalently. The discovery and progress of these new agents, as well as the discovery of compounds with unique mechanisms of action, will be the subject of this review. This subject was last reviewed in this series in 1990; compounds discussed in that chapter which continue to show promise in clinical trial will be updated briefly but structures will not be repeated herein (1). The majority of this chapter will focus on promising new agents that have entered clinical trial in the past few years, preclinical compounds with a degree of efficacy in animal models that would appear to warrant development to clinical trial, and compounds that demonstrate unique mechanisms or unusual activities in preclinical studies.

Topoisomerase I Inhibitors – Analogs of camptothecin were highlighted as promising new candidates in our previous review of antineoplastic agents (1). Topotecan and CPT-11 have now advanced to extensive Phase II trials. Both agents are limited by myelosuppression which is of relatively brief duration and is not cumulative. CPT-11 produces severe diarrhea as a major side-effect, but this has not been a problem with topotecan. Activity has been reported for one or both of these agents in lung, gastrointestinal, gynecologic and hematologic malignancies (2). A third analog, 9-aminocamptothecin, has just started Phase I studies but is limited by poor solubility.

The exceptional preclinical profiles and apparent clinical efficacy of compounds related to camptothecin has spawned a search for other classes of compounds that produce DNA damage mediated by topoisomerase I (topo I). Two structural classes have been described as dual inhibitors of topo I and topo II; saintopin (**1**) was identified in a natural product screen for topoisomerase inhibitors (3) and intolpicin (**2**) emerged from synthesis of compounds related to ellipticine (4). Both of these agents are capable of stabilizing the cleavable enzyme-DNA complex with either purified topo I or topo II. It has not been established whether the cytotoxic effects of these agents in whole cells is the result of both or one of these activities. Intolpicin is in early clinical trial (5) while solubility problems have impeded the development of saintopin.



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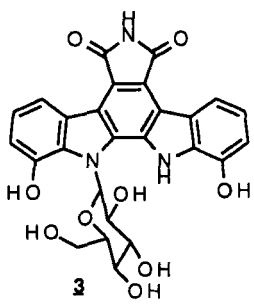


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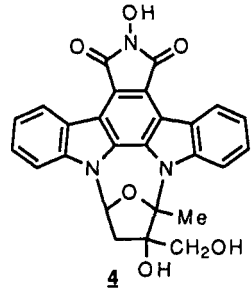
Indolocarbazole microbial metabolites (e.g., staurosporine) have been known for some time as potent inhibitors of protein kinase C (6). Certain derivatives of this structural class have recently been identified which demonstrate antitumor activity and inhibit topo I in a fashion similar to camptothecin, e.g., stabilize the enzyme-cleaved DNA covalent intermediate (7, 8). The most potent topo I inhibitory indolocarbazoles are ED-110 (**3**) and KT6528 (**4**). These analogs appear to be DNA intercalators, and related compounds that bind to DNA less strongly are weaker inhibitors of topo I. Thus, there have now been described DNA binding agents that induce cleavable complexes selectively through inhibition of either topo I or topo II, as well as DNA binders that inhibit both enzymes nonselectively.

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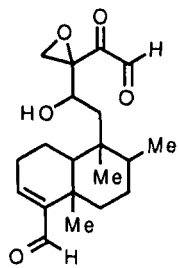


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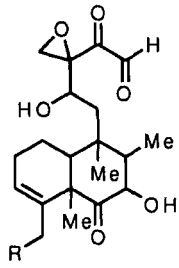
Topoisomerase II Inhibitors – Several clinically useful antineoplastic agents inhibit DNA topo II. Among inhibitors of this important enzyme, three compound classes discussed in a previous review (1) have shown promise in ongoing clinical trials: anthrapyrazoles, ellipticines, and amonafide. The anthrapyrazoles DuP 937, DuP 941 (biantrazole), and DuP 942 (piroxantrone) are intercalative DNA topo II inhibitors designed on the basis of combining the activity of the anthracyclines and anthracenediones with decreased cardiotoxicity. The mechanism of action of these agents was reviewed recently (9). Of the three anthrapyrazoles in clinical trial, biantrazole has shown the most promise to date, demonstrating a 63% response rate in a phase II clinical trial for breast cancer (10). Earlier in clinical development are piroxantrone and DuP 937, which has also shown activity in breast cancer but not in gastrointestinal or lung cancer (11-14).

The most promising ellipticine analogs are datelliptium and retelliptine (15). Datelliptium demonstrated objective responses in previously treated breast cancer and has fewer of the cholinergic side effects previously reported with elliptinium acetate (16). Mechanistic studies suggest that datelliptium, which demonstrates increased cytotoxicity *in vitro* compared to related analogs, produces DNA damage that is more persistent than that produced by ellipticine and elliptinium (17). Retelliptine, another water-soluble analog earlier in clinical development, appears to share some of the toxicities observed with earlier ellipticine analogs (18). Amonafide, a DNA intercalator that stabilizes topo II-mediated DNA strand breaks, demonstrated activity against advanced breast cancer in clinical trial with dose-limiting myelosuppression (19). The N-acetyl metabolite of amonafide, which is formed at different rates among individuals, is more active than the parent (20); thus amonafide demonstrates variable toxicity among patients.

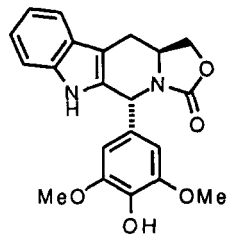
In light of the success of topo II inhibitors, particularly in breast cancer, significant effort has gone into the discovery and design of new inhibitors in the last few years. A screening program based on the inhibition of topo II *in vitro* led to the discovery of clerocidin (5), terpentecin (6), and UCT4B (7), microbial terpenoids that induce heat-stable DNA-topo II complexes but do not bind to DNA (21, 22). Design of a new topo II agent, azatoxin (8), was accomplished by combining certain structural features of the known inhibitors etoposide and ellipticine (23). Azatoxin induces enzyme-mediated DNA breaks, inhibits the catalytic activity of topo II, and does not intercalate into DNA. SAR with respect to substitution in the pendant ring of azatoxin were similar to those observed for etoposide.



5

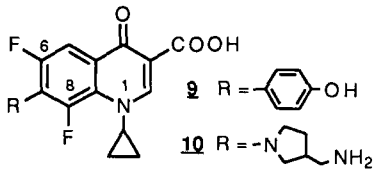


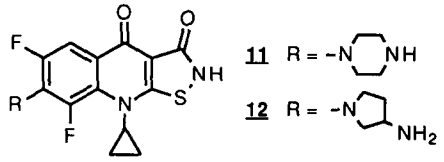
6 R = H
7 R = OH



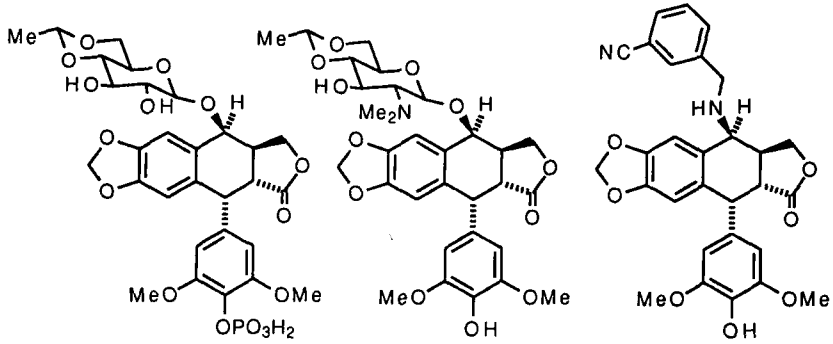
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The bacterial topo II, DNA gyrase, is the target of the antibacterial quinolones. It was thought previously that compounds of this class were specific for the bacterial enzyme; however, quinolones that inhibit eukaryotic topo II were identified recently by screening against the mammalian enzyme *in vitro* (24-27). A fluorine substituent at the 8-position was found to increase the potency of quinolone derivatives against eukaryotic topo II, as demonstrated by comparing potency of CP-115,953 (**9**) to that of its congener lacking an 8-fluoro group (24). Studies of the cytotoxicity of **9** in yeast cells demonstrated that topo II is its primary target (25). In another study, ten compounds active against topo II were found by screening 90 quinolones, and compound **10** demonstrated modest antitumor activity in mice (26). Antibacterial isothiazoloquinolones **11** and **12** were also found to be active against the eukaryotic enzyme (27). In each of these studies, the most potent analogs contained halogens at positions 6 and 8 and a cyclopropyl group at N-1.



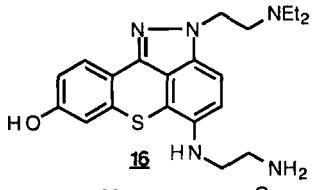


Among analogs of existing topo II inhibitors, considerable effort has been expended in the area of demethylepipodophyllotoxins. A water-soluble orally active prodrug, etopophos (**13**) has advanced to clinical trial (28, 29). Etopophos demonstrated high but variable oral absorption and was converted to etoposide in patients. NK611 (**14**), a water-soluble analog containing an amino-substituted sugar, inhibits topo II with potency comparable to etoposide and has higher oral absorption in dogs (30, 31). NK611 demonstrated dose-limiting leukopenia in initial clinical trial and appears to be about 3-fold more potent than etoposide. Analogs of etoposide containing an anilino or benzylamino group in place of the glycoside moiety, as exemplified by **15**, have also been reported to have good preclinical efficacy (32). SAR in this series indicates that an 4β-anilino or 4β-benzylamino ring system is required for topo II inhibition.

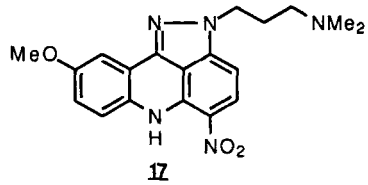


13 **14** **15**

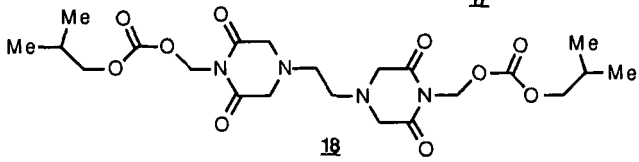
Intercalative topo II inhibitors related in structure to mitoxantrone and the anthrapyrazoles include the benzothiopyranoindazole CI-958 (**16**) and the pyrazoloacridine PD 115934 (**17**). CI-958 has an excellent preclinical profile and is currently in early clinical trial (33). In the pyrazoloacridine class, PD 115934 was selected for clinical evaluation based on selective activity against solid tumor cell lines *in vitro* as well as good activity in several experimental tumor models *in vivo* (34, 35). Sobuzoxane (**18**) is an analog of razoxane that inhibits topo II without stabilizing the cleavable protein-DNA complex; this compound class does not bind to DNA. Sobuzoxane has demonstrated activity against adult T-cell leukemia and non-Hodgkin's lymphoma in clinical trials in Japan (36).



16



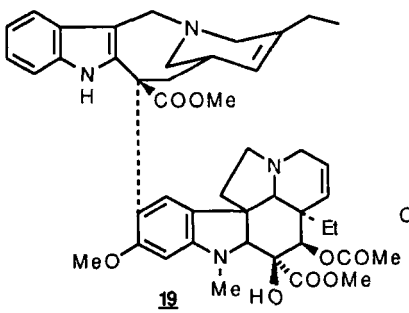
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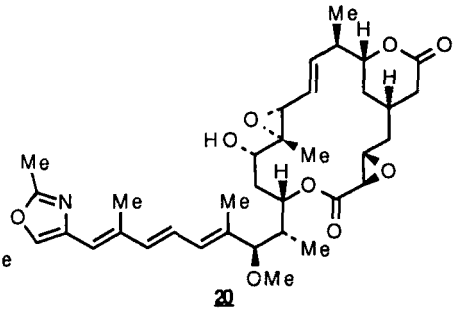
18

Antimitotic Agents – One of the more significant recent advances in cancer chemotherapy is the development and subsequent approval of taxol for the treatment of ovarian cancer. The rapid approval of taxol was based on the impressive activity observed in clinical trials with responses observed in cancers of the ovary, breast, and lung, and in melanoma (37, 38). Taxotere, a semi-synthetic taxol analog with increased water-solubility, has also demonstrated impressive activity in early clinical trials with a similar toxicity profile to that of taxol (39, 40). Both drugs promote the assembly of microtubules and inhibit their depolymerization (41).

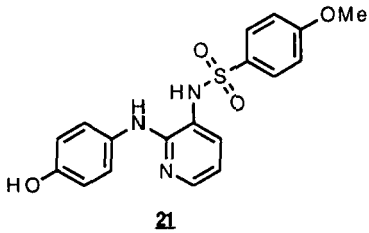
Vinorelbine (navelbine, **19**) is a semi-synthetic derivative of the *Vinca* alkaloids that acts by preventing microtubule formation (42). Preclinical studies demonstrated that vinorelbine has broader antitumor activity with lower toxicity than vinblastine and vincristine. In clinical trials in advanced breast cancer, vinorelbine demonstrated 40-50% response rates when given as first-line treatment (43). Vinorelbine also produced responses in nonsmall cell lung cancer and is a promising new antineoplastic agent (44). Another antimitotic agent that binds to the *Vinca* domain on tubulin is rhizoxin (**20**), a compound isolated from the fungus *Rhizopus chinensis* which is currently in early clinical trial (45, 46). Tubulin binders in preclinical development are E7010 (**21**), a sulfonamide that inhibits tubulin polymerization and appears to differ in mechanism from other antimitotic agents (47-49), and SQZ (**22**), which binds to the colchicine site on tubulin and is reported to have good activity in animal tumor models (50).



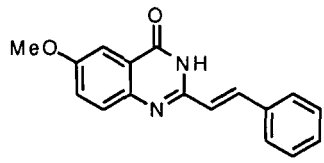
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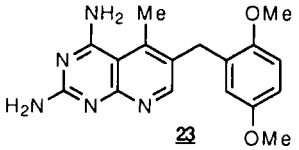
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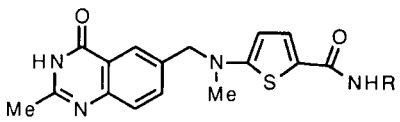
22

Antimetabolites – New antimetabolites that interfere with nucleic acid biosynthesis continue to be of major interest in oncology. Two compounds described in Volume 25 of this series have made significant progress in clinical development in the past two years (1). Gemcitabine (2'-difluoro-2'-deoxycytidine) is currently in Phase II trial in a broad spectrum of solid tumors and has produced objective responses in small cell and nonsmall cell lung cancers, pancreatic cancer, head and neck tumors, breast and ovarian cancers (51-53). Lometrexol (DDATHF) is an antifol with a novel mechanism – inhibition of GAR transformylase (54). Clinical studies of lometrexol have been slowed by cumulative and dose-limiting stomatitis and thrombocytopenia (55). Preclinical observations of selective protection of normal tissues by leucovorin (56) have led to clinical evaluation of the combination with major escalation of lometrexol dose (57).

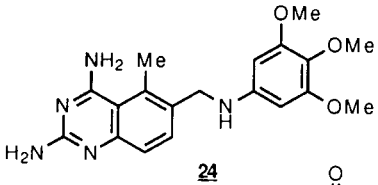
Several novel and promising antifols with diverse mechanisms of action are currently in development. Lipophilic dihydrofolate reductase (DHFR) inhibitors, piritrexim (**23**) and trimetrexate (**24**) have been in clinical study for several years and have shown some evidence of efficacy but no convincing advantage over methotrexate (58). There is considerable interest in another DHFR inhibitor, edatrexate (**25**), which has shown activity in nonsmall cell lung cancer (59) and is in broad Phase II testing. Three antifols that are selective inhibitors of thymidylate synthase (TS) are in development – ICI D1694 (**26**), which has a K_i of 60 nM for TS (60), is in Phase I testing (61), and more potent competitive TS inhibitors, 1843U89 (**27**) and LY231514 (**28**), have been shown recently to have excellent activity in preclinical tumor models (62, 63). Crystallographic data and computer-assisted drug design led to the development of novel and potent benz[cd]indole inhibitors of TS (64), as exemplified by **29**. New nucleoside derivatives that have been described as having significant efficacy in preclinical models include (E)-2'-deoxy-2'-fluoromethylenecytidine (FMdC, **30**), which inhibits ribonucleotide reductase, and 2'-deoxy-2'-methylenecytidine (DMDC, **31**), which has an unknown mechanism (65, 66).



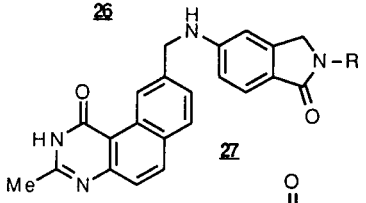
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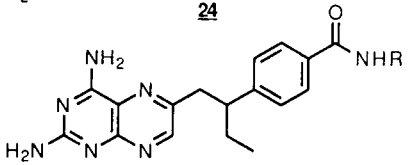
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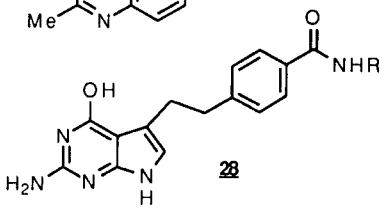
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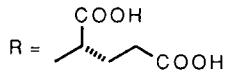
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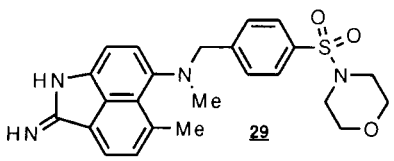


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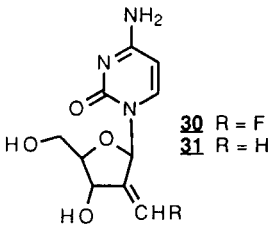


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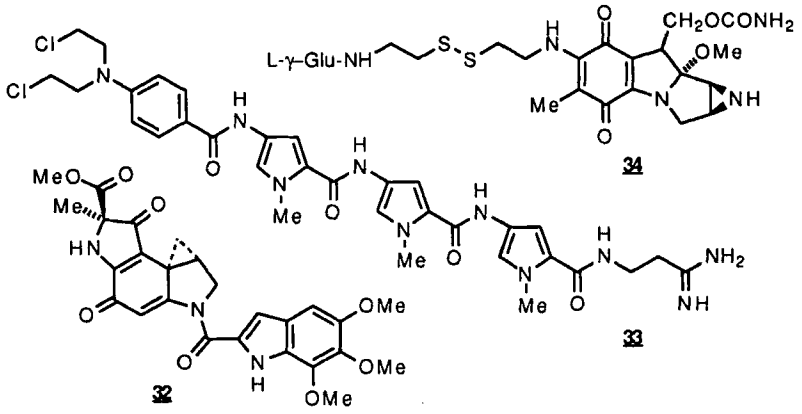
29



30 R = F
31 R = H

DNA Interactive Agents – DNA interactive agents, particularly those that bind covalently in the minor groove or cleave the backbone, have continued to show promise as antineoplastic agents. Three such agents discussed in Volume 25 of this series are the cyclopropylpyrroloindole (CPI) analogs related to the natural product CC-1065 – adozelesin, bizelesin and carzelesin (1). Each of these agents binds in the minor groove of DNA and forms a covalent adduct with the N3 of adenine. Adozelesin is the farthest advanced of these agents, having recently entered Phase II clinical trial (67, 68). The prodrug carzelesin and the dimer bizelesin have demonstrated impressive preclinical activity and are in development (69, 70).

The duocarmycins and pyrindamycins (e.g. duocarmycin A, **32**) are natural product CPIs (71); a series of analogs have been prepared and studied *in vitro* (72). Another minor groove alkylating agent was synthesized by appending a reactive moiety onto distamycin. FCE 24517 (**33**), currently in clinical trial, forms covalent adducts with DNA and demonstrated broad spectrum efficacy in preclinical tumor models (73, 74). A water-soluble mitomycin analog, KW 2149 (**34**), was prepared and shown to lack cross-resistance against mitomycin C-resistant cell lines; it is currently in clinical trial (75). An orally active analog of chartreusin, IST-622 (**35**), has completed Phase I clinical trial in Japan with granulocytopenia as the dose-limiting toxicity (76).

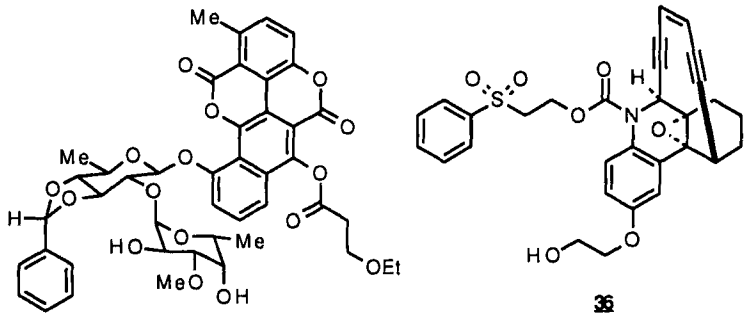


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32

A series of new DNA cleaving molecules based on the reactive moiety in enediyne anticancer antibiotics (calicheamicins, esperamicins, and dynemicins) was designed and synthesized (77, 78). Simplified enediyne-type compounds such as **36** damage DNA upon activation by chemical or biological means and are extremely potent cytotoxic agents *in vitro*.

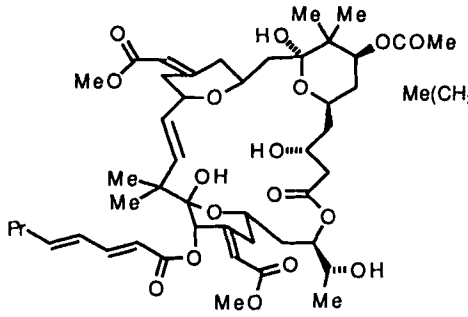


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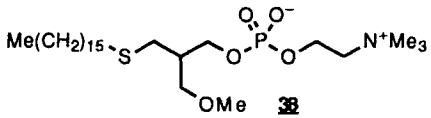
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Miscellaneous – Much of the focus in anticancer drug discovery has been directed away from agents that kill cells by producing DNA damage and toward modulators of signal transduction pathways that have become unregulated or otherwise aberrant in malignant transformation. In our previous review, a number of signal transduction inhibitor leads were described; none of these has yet demonstrated clinical utility (1). However, clinical trials have been initiated on the potent

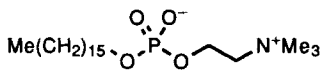
partial agonist of protein kinase C, bryostatin (37), isolated from a marine bryozoan (79). There has been considerable interest in glycerol lipid/PAF analogs as potential anticancer agents (80). Examples include ilmofosine (38) and miltefosine (39), which have been widely studied in preclinical models and clinical trials (80, 81). It is unclear whether the cytotoxicity of these lipids is due to interference with signal transduction pathways or with membrane function; the clinical utility of this class of compounds has yet to be established.



37

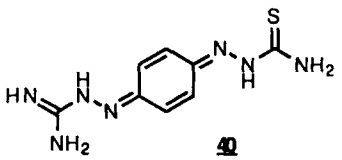


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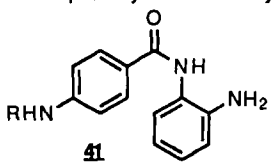


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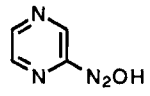
There are a number of agents with either novel or unknown mechanisms of action that are currently being evaluated as anticancer agents. Ambazone (40) has been the subject of a number of publications from Germany and is reported to be in clinical trial (82, 83). The mechanism by which this relatively simple compound acts is not known. Another uncomplicated structure of considerable interest is dinaline (41, R=H) and its derivatives GOE 5549 (R=Ac) and GOE 4902 (R=Me). These compounds have good activity in solid tumor models due to an as yet undefined mechanism (84). Pyrazine diazohydroxide (42) is a third small molecule lead with unknown mechanism developed based on activity in animal tumor models; Phase I clinical trials have shown the drug to be limited by delayed and possibly cumulative myelosuppression (85).



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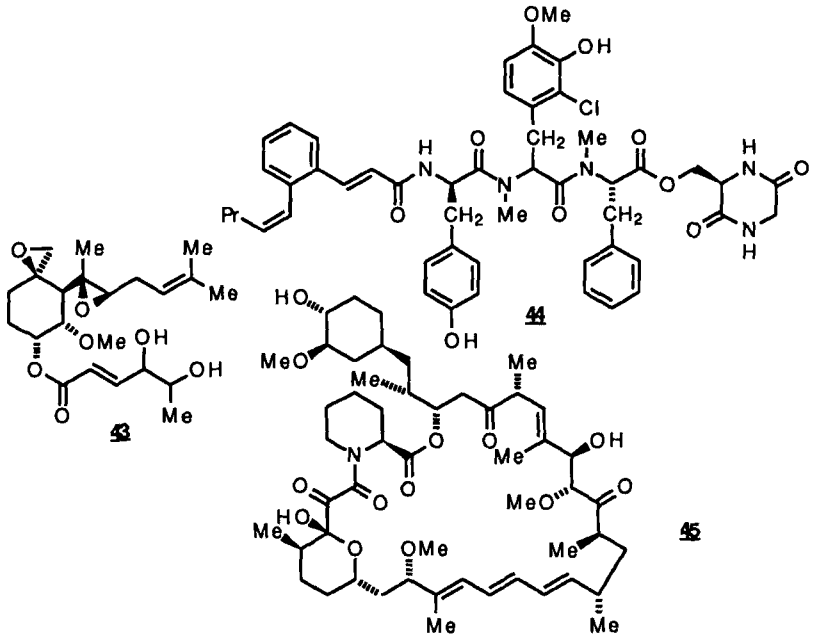
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Mechanism-based screens for natural products have identified some interesting antineoplastic leads during the past year. The observation that the antibiotic fumagillin and related derivatives interfere with tumor-induced neovascularization will lead to the initial clinical test of the concept of inhibiting angiogenesis as a means of cancer treatment (86). FR-111142 (43) is reported to be both less toxic and more active than fumagillin and is a candidate for development (87). A potent inhibitor (IC_{50} = 0.3 μM) of protein farnesylation, pepticinnamin E (44) has been described (88). It will be of great interest to see if this novel microbial product demonstrates significant activity (or selectivity) in tumors with mutant *ras*. Rapamycin (45) is an antifungal antibiotic discovered almost 20 years ago (89) that is known to have an interesting spectrum of antitumor activity in preclinical tumor models (90). This compound is of greater interest currently as a potential immunosuppressant (90). During the past year the mechanism of action of rapamycin has been identified. Although not yet completely defined, it appears that rapamycin inhibits the p70 S6 kinase and cyclin-dependent protein kinase pathways involved in regulation of cell cycle traverse (91-93). This is the first example of an inhibitor of cell cycle regulation; it is of considerable interest that this inhibition is associated with activity in solid tumor models.

Conclusion – Significant advances in the field of antineoplastic chemotherapy have occurred over the last few years. With the approval of the new antimitotic agent taxol and evidence for clinical efficacy for several promising new agents, the 1990's are likely to be a decade of progress against several types of cancer. Interestingly, the anticancer agents currently showing promise in



clinical trials operate by mechanisms similar to those of established drugs, i.e. DNA damage induction (including topo I and II inhibitors), interference with microtubule function, and inhibition of nucleic acid biosynthesis. Several newer compounds have improved side-effect profiles and increased efficacy (or activity against refractory tumors) compared to existing therapies. Antineoplastic agents with completely novel mechanisms of action are the subject of intensive preclinical study and may begin to impact on cancer treatment towards the end of the decade.

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SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

Editor: William K. Hagmann
Merck Research Laboratories, Rahway NJ 07065-0900

Chapter 19. Growth Hormone Secretagogues

William R. Schoen, Matthew J. Wyvratt, Jr. and Roy G. Smith
Merck Research Laboratories, P.O. Box 2000
Rahway, New Jersey 07065-0900

Introduction - During the past decade, pituitary hormones have been the subject of intense research, both from a molecular approach as well as clinically. All of the pituitary hormones, growth hormone (GH), luteinizing hormone (LH), follicle stimulating hormone (FSH), adrenocorticotropic hormone (ACTH), thyrotropin stimulating hormone (TSH) and prolactin (PRL) have been thoroughly studied with respect to structure, function and regulation (1-5). While secretion of pituitary hormones is a complex process that is influenced by a variety of factors, both hormonal and behavioral, the principal regulator is a corresponding hypothalamic releasing factor or secretagogue. The endogenous secretagogues that modulate the secretory pathways have also provided attractive targets for basic and clinical research. With the evolution of modern recombinant DNA technology, human growth hormone has become more widely available. In addition to replacement therapy in GH deficient states, a wide variety of other indications have now been considered as candidates for GH treatment. Expanding interest in clinical applications of GH has been accompanied by accelerated efforts in basic research on the hormone and its regulation. This chapter will focus on recent advances in basic and clinical aspects of GH, its endogenous secretagogue, GRF, and other agents that stimulate GH release.

GROWTH HORMONE

Regulation - The secretion of GH from the pituitary gland is controlled by a complex neuroendocrine system that in turn regulates the release of two hypothalamic hormones, growth hormone releasing hormone/factor (GHRH, GRF) and somatostatin (1) (Figure 1). Both hormones act on the GH containing cells in the pituitary gland, the somatotrophs. GRF positively regulates GH release while the tetradecapeptide somatostatin is inhibitory (Figure 2). The combined effects of GRF and somatostatin produce a pulsatile pattern of GH release which is characterized by pronounced circadian variation (6). GRF also stimulates adenylate cyclase activity resulting in increased rates of synthesis of GH mRNA and activation of L-type calcium channels (7-9). In contrast, somatostatin inhibits adenylate cyclase activity and causes a hyperpolarization of somatotrophs resulting in inhibition of GH release (10). Thyroid hormone, glucocorticoids and retinoic acid also act on somatotrophs to synergistically increase GH gene expression (1). Agents that stimulate kinase A or kinase C pathways in somatotrophs or compounds causing membrane depolarization also activate L-type calcium channels resulting in GH release (9,10).

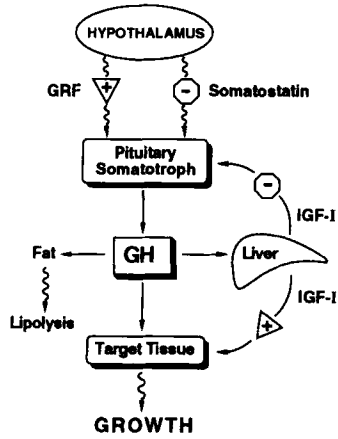


Figure 1. Regulation of GH release by the Pituitary-Hypothalamus Axis

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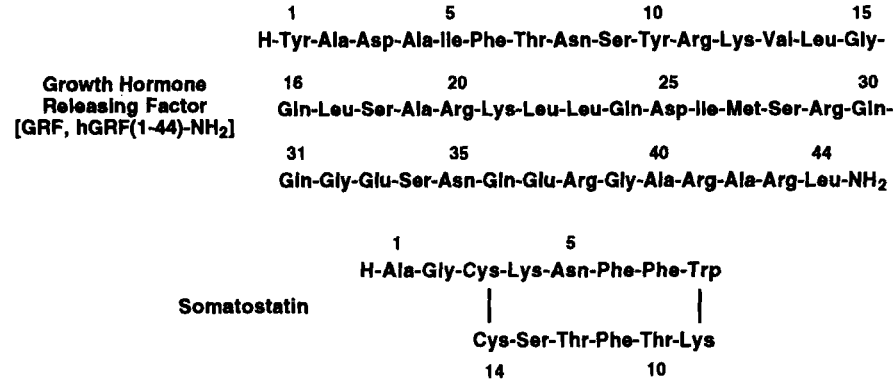


Figure 2. Primary Regulators of Growth Hormone Release

In addition to well documented growth promoting action, GH accelerates other metabolic processes such as lipolysis and protein synthesis (11). Anabolic effects of GH are mediated through two major pathways, (i) directly, by activation of GH receptors located in target tissues; and, (ii) indirectly, through the actions of insulin-like growth factor I (IGF-I), which is released from the liver in response to GH stimulation. Structurally, IGF-I is similar to insulin and both hormones act through similar receptors (12). IGF-I elicits a slow, growth-promoting response in tissues in addition to acute, insulin-like effects. Feedback regulation by IGF-I and GH at the hypothalamic and pituitary levels inhibits GH release (13).

GH Receptor Studies - The GH receptor is expressed on the surface of a number of different cell types, and binding of GH results in stimulation of growth of muscle, bone and cartilage (14). The receptor is organized into three domains comprising an extracellular hormone binding domain, a single transmembrane segment and an intracellular domain. Interestingly, a GH binding protein consisting of residues 1-246 of the extracellular domain is present in serum (15). Presumably, the binding protein is involved in the regulation of GH clearance. The crystal structure of the human GH receptor complex with GH has been reported (16). Surprisingly, the 2.8 Å crystal structure shows that the complex consists of one molecule of GH per two molecules of receptor. In each receptor monomer essentially the same residues participate in ligand binding, yet the two binding sites on GH have no structural similarity. Substantial contact occurs between the carboxyl-terminal domains of the GH-receptor complex implying that hormone-dependent dimerization may be important for signal transduction (16).

Structural homologies exist between the extracellular domains of the GH receptor and receptors for interleukins, granulocyte and granulocyte macrophage stimulatory factors and erythropoietin (17,18). While the signal transduction mechanism for these receptors is unclear, the binding sequence and events leading to mediation of a biological signal appear to be similar to the sequential dimerization model proposed for GH receptor signal transduction (19). Accordingly, a hybrid receptor has been constructed where the extracellular domain of the GH receptor is linked to the transmembrane and intracellular domains of the granulocyte colony stimulating factor receptor (20). Expression of this hybrid receptor in the FDC-P1 myeloid leukemia cell line results in GH-dependent dimerization and proliferation of the cells (20). Subsequent studies involving a variety of granulocyte colony stimulating factor receptor (G-CSF-R) and GH receptor chimeras show that although the transmembrane and cytoplasmic domains of the G-CSF-R and GH receptor have no obvious similarities, their regions are interchangeable as defined by signal transduction resulting from receptor dimerization in FDC-P1 cells (21). Studies in transgenic mice show that over-expression of a GH receptor containing mutations which would predictably inhibit receptor dimerization produces a dwarf phenotype (22). Thus, the results from X-ray crystallography, mutagenesis and chimeric receptor studies, and transgenic mice expressing mutant GH receptors, are consistent with the notion that receptor dimerization is essential for the biological actions of GH.

In addition to its effect on increasing plasma levels of IGF-I, GH activates expression of the early response genes c-*fos* and c-*jun* (23). Although the growth stimulatory effects of GH are, in

part, mediated by IGF-I, increases in c-*fos* and c-*jun* expression occur in the absence of an IGF-I response. Therefore, certain physiological effects of GH may be manifested in the absence of increased circulating levels of IGF-I. The induction of the early response genes is consistent with transcriptional activation of other genes that are important in the longer term effects of GH on differentiation and metabolism.

GH - Clinical Evaluation - Prior to the availability of recombinant GH in the mid-1980's, hormone deficiency was treated clinically using material isolated from pituitary glands of cadavers. This source was relatively limited and was not always completely pure. Indeed, Creutzfeldt-Jakob disease has occurred in some individuals treated with cadaver-derived GH (24). Accordingly, the use of exogenous GH had been limited to situations where a suitable alternative was not available, such as in GH deficient children. Following the large scale manufacture of recombinant human GH, potential uses of GH have expanded to include other therapeutic areas. In addition to GH deficient children (25), GH has been evaluated clinically in GH deficient adults (26). GH treatment has also been shown to be beneficial in the treatment of severe burn victims (27), in Turner's syndrome (28), for reversing the adverse effects of glucocorticoid treatment (29) and for treating muscle and exercise tolerance deficiencies in GH deficient adults (30). Preliminary results indicate that GH treatment may be effective in preventing osteoporosis (31). T cell development is accelerated by GH, suggesting a possible role in treatment of T cell deficiencies (32). Other potential indications for GH therapy include ovulation induction (33), chronic renal insufficiency (34) and use in renal transplantation (35).

Clinical evaluation of GH in the elderly population has recently shown encouraging results. It is known that with advancing age, spontaneous GH secretion decreases even in the absence of pituitary dysfunction (36). The resulting decline in GH-stimulated lipolysis and protein synthesis has been related to decreases in lean body and bone mass, and increases in body fat. GH replacement therapy in elderly men has produced dramatic effects in partially reversing these age-related trends and significant improvements in muscle tone, skin tone and bone mass have been observed (37). Clinicians have noted that "the effects of six months of human growth hormone on lean body mass and adipose-tissue mass were equivalent in magnitude to the changes incurred during 10 to 20 years of aging" (37). An expanded study will investigate the effects of GH treatment in combination with physical exercise in aged subjects (38).

Alternatives to GH Treatment - As a large protein (191 amino acids), GH lacks oral bioavailability and must be administered parenterally, usually on a daily basis. This route of administration does not mimic normal physiology where GH secretion occurs in discrete circadian pulses. An alternative to parenteral administration of GH is treatment with a GH secretagogue, ideally orally active, to stimulate release of endogenous GH from the pituitary gland. In this manner, the natural pulsatile profile of GH secretion could be simulated through multiple dosing of an orally active agent. Since the identification of the endogenous GH secretagogue, GRF, a substantial amount of research has been directed towards identifying smaller structures that mimic its action and are potentially orally absorbed.

GROWTH HORMONE RELEASING FACTOR

The discovery of human growth hormone releasing factor (hGRF) in 1982 (39,40) as the primary hypothalamic hormone responsible for release of growth hormone from pituitary somatotrophs has stimulated intense research on this class of secretagogue (41). While three GRF's were initially characterized from patients with acromegaly, the amidated 44-amino acid peptide, hGRF(1-44)-NH_2, has been established as the primary hormone in man (Figure 2). Growth hormone releasing factors isolated from pig, goat, cow, sheep, rat and mouse sources show a high degree of homology with hGRF (41,42). The receptor for hGRF has recently been cloned (43). The cDNA encodes a 423-amino acid receptor that has seven transmembrane spanning domains characteristic of G-protein coupled receptors. It is a member of the secretin family of G-protein coupled receptors and there is sequence homology between hGRF and intestinal peptides of the secretin-glucagon family (43,44).

Structure-Activity Relationships - The early structure-activity relationships established for GRF clearly indicate that the N-terminus portion of the peptide is critical while the C-terminus does not contribute greatly to bioactivity (41). The C-terminus shortened peptide GRF(1-29)-NH_2 is the minimum structure which maintains the full intrinsic activity and potency of GRF(1-44)-NH_2. The peptidyl region responsible for intrinsic biological activity is localized between amino acid residues 4 and 20 (45). Because of its simplified structure and synthesis, most of the structure-activity relationships on growth hormone releasing factors have been developed for the truncated fragment,

GRF(1-29)-NH_2. A good correlation between GRF receptor binding affinity *in vitro* and GH-releasing activity in the rat pituitary cell assay has been reported (46,47).

Human GRF(1-44)-NH_2 is rapidly cleaved at the Ala^2-Asp^3 bond *in vivo* in humans ($t_{1/2}$ = 6.8 min.) by the hydrolase, dipeptidyl peptidase IV (DPP IV), to generate the biologically inactive fragment GRF(3-44)-NH_2 (48-50). GRF(1-44)-NH_2 is also degraded at the Arg^{11}-Lys^{12} bond to a lesser extent by trypsin-like enzymes (49). The shortened peptide, GRF(1-29)-NH_2 is likewise cleaved by DPP IV to give a biologically inactive GRF(3-29)-NH_2 fragment (49,51). A major focus of research on GRF's has been to identify analogs with enhanced enzymatic stability which would perhaps have greater clinical utility. An initial approach is to modify, through the incorporation of *D*-amino acids at positions 1-3, the N-terminus of GRF(1-29)-NH_2 to decrease enzymatic susceptibility. [*D*-Ala^2]-GRF(1-29)-NH_2 is completely resistant to metabolism by DPP IV (49) and is approximately 50-fold more active than GRF(1-29)-NH_2 in rats and pigs (52). Substitution of *D*-Arg at position 2 in GRF(1-29)-NH_2 produces an antagonist of GRF (53). Since dipeptidyl peptidase IV requires a free amino group at the N-terminus, the acetylated derivative [N-Ac-Tyr^1]-GRF(1-29)-NH_2 (52) and the des-amino derivative [desNH_2Tyr^1]-GRF(1-29)-NH_2 (49) are more resistant to DPP IV proteolysis and, therefore, have greater biological activity than GRF(1-29)-NH_2. Addition of a proline residue to the N-terminus of the native protein, as in [Pro^0]-GRF(1-44)-NH_2, has also been claimed to increase metabolic stability *in vivo* (54). Incorporation of a reduced peptide bond isostere (-CONH- to -CH_2NH-) into the peptide backbone at the 1-2, 2-3 or other positions of GRF(1-29)-NH_2 resulted in a loss of most GH-releasing activity (55).

Conformational analyses of GRF(1-29)-NH_2 indicate that the peptide has a high degree of α-helical and amphiphilic character (56-58). An additional approach to increasing biological potency and resistance to enzymatic degradation is to optimize its α-helical and amphiphilic nature and thereby, hopefully, mimic the preferred bioactive conformation. Replacement of Gly^{15} in GRF(1-29)-NH_2 with more hydrophobic amino acids such as Ala was predicted to extend the α-helical region and maximize its amphiphilic structure, whereas Sar, generally considered to disrupt an α-helix, would decrease activity. [Ala^{15}]-GRF(1-29)-NH_2 was found to be approximately 4-fold more potent in the rat pituitary cell assay than GRF(1-29)-NH_2 while [Sar^{15}]-GRF(1-29)-NH_2 had greatly diminished bioactivity (59). The greater binding affinity of [Ala^{15}]-GRF(1-29)-NH_2 and the lack of binding affinity of [Sar^{15}]-GRF(1-29)-NH_2 toward the GRF receptor (46) suggest that [Ala^{15}]-GRF(1-29)-NH_2 more closely mimics the preferred bioactive conformation. Structural modifications can also be combined to generate potent analogs, e.g., [desNH_2Tyr^1, *D*-Ala^2, Ala^{15}]-GRF(1-29)-NH_2 is five-fold more potent than GRF(1-29)-NH_2 as a GH secretagogue (60) and [*D*-Arg^2, $Ala^{8,15}$]-GRF(1-29)-NH_2 is one of the most potent antagonists of GRF(1-29)-NH_2 reported to date (53). Potential bioactive conformations of linear peptides can also be mapped through the synthesis of various cyclic analogs. Cyclic versions of GRF(1-29)-NH_2 have been identified which are more potent GH secretagogues than GRF(1-29)-NH_2 (59-61). Cyclo^{8,12}[*D*-Ala^2, Asp^8, Ala^{15}]-GRF(1-29)-NH_2 and Cyclo^{8,12}[N-MeTyr^1, *D*-Ala^2, Asp^8, Ala^{15}]-GRF(1-29)-NH_2 are both 3-4 fold more active than GRF(1-29)-NH_2 (61). Conformational analyses confirm that these cyclic peptides have greater α-helical character than the linear peptides (61). Related cyclic analogs have also been shown to be more resistant to DPP IV enzymatic degradation (50).

GRF - Animal Growth Studies - Growth hormone plays an important role in the regulation of growth in livestock and in the partitioning of nutrients between muscle and adipose tissue. Exogenous GH has been shown to improve the growth and carcass parameters in swine performance trials (62). GRF(1-44)-NH_2 (63), GRF(1-29)-NH_2 (63), and [desNH_2Tyr^1, *D*-Ala^2, Ala^{15}]-GRF(1-29)-NH_2 (64,65) have been evaluated in pigs by subcutaneous administration. In general they are as effective as porcine GH in improving growth rate, feed efficiency and carcass characteristics.

GRF - Clinical Studies - Early clinical studies with GRF(1-44)-NH_2 and GRF(1-29)-NH_2 in normal individuals at doses ranging from 0.1 to 10 μg/kg, given intravenously, produced significant stimulation of GH secretion. The administered drugs are specific for release of GH except for occasional slight elevation in PRL levels. Transient facial flushing at high doses of GRF's have also been observed. The majority of children with idiopathic growth hormone deficiency have a hypothalamic defect; response to exogenous GRF has been employed in this group as a diagnostic tool to differentiate hypothalamic from pituitary dysfunction (66). Approximately 75-80% of these patients respond to an intravenous bolus of GRF. While the majority of GH deficient children experience growth acceleration with GRF treatment, the results are not comparable to that

observed in patients treated with GH (67,68). A number of superactive analogs, e.g., [*D*-Ala^2]-GRF(1-29)-NH_2 (69), [Nle^{27}]-GRF(1-29)-NH_2 (70) and [Ac-*D*-Tyr^1, *D*-Ala^2]-GRF(1-29)-NH_2 (71) have also been tested in humans. Unfortunately, the high activity observed with these analogs in animal models does not translate to significantly greater efficacy in humans. Subcutaneous and intranasal routes of administration of GRF analogs have also been examined in humans; however, higher drug doses (10-300 fold) are required to achieve efficacy comparable to that achieved by intravenous administration (70,71). GRF(1-29)-NH_2 has also been reported to reverse the age-related decreases in GH and IGF-I in healthy old men when subcutaneously dosed for fourteen days (72). This suggests that prolonged treatment with GRF may improve age-related alterations in body composition (73).

PEPTIDYL SECRETAGOGUES

In addition to GRF, several other structurally distinct peptidyl compounds have been reported to release GH. MB-35, a 35 amino acid peptide isolated from calf thymus, has been shown to release GH and PRL *in vitro* through hypothalamic stimulation (74). GH release by the 29-amino acid neuropeptide galanin is also mediated through the hypothalamus; studies *in vitro* show that both GRF and somatostatin are released from isolated hypothalamic tissues (75). Pituitary adenylate cyclase-activating polypeptide (PACAP), a 38-amino acid neuropeptide isolated from ovine hypothalamus, releases GH *in vitro* in a dose-dependent manner (76). PACAP lacks specificity for GH and release of other pituitary hormones is observed. The nonapeptide, delta sleep-inducing peptide (DSIP), isolated from rabbit cerebral venous blood, shows a dose-dependent GH response in rats, mediated hypothalamically through dopaminergic stimulation (77). The potent octapeptide vasoconstrictor, angiotensin II, also releases GH and PRL *in vitro* (78).

Growth Hormone Releasing Peptides - Of the peptidyl secretagogues, the most extensively studied have been the synthetic methionine-enkephalin derived growth hormone releasing peptides (GHRP's) identified by Bowers (41). Historically, the original pentapeptide lead, Tyr-*D*-Trp-Gly-Phe-Met-NH_2, was identified *prior* to the isolation and characterization of the endogenous secretagogue GRF. Although bearing no structural homology to GRF, compounds in this series act directly on the pituitary to release GH in a dose-dependent fashion; other pituitary hormones LH, FSH, TSH, PRL and ACTH are unaffected (79). Extensive structure-activity work has demonstrated the importance of an aromatic *D*-amino acid residue at position 2 of the peptide (80). Enhanced potency *in vitro* is achieved by replacement of the Phe^{4}Met^5 segment with Trp^4*D*-Phe^5. It has been proposed that interaction of the aromatic amino acid residues at positions 2, 4 and 5 through π-stacking plays a key role in determining an active conformation. A pivotal finding was that appending an amidated lysine residue to the C-terminus confers good activity *in vivo* to these molecules. The hexapeptide GHRP-6 (**1**) is one of the more widely studied analogs is this series (81). A more potent second generation heptapeptide, GHRP-1 (**2**), containing a *D*-(β-naphthyl)-alanine replacement for *D*-Trp, has recently been described (82).

His-*D*-Trp-Ala-Trp-*D*-Phe-Lys-NH_2 **1**

Ala-His-*D*-βNal-Ala-Trp-*D*-Phe-Lys-NH_2 **2**

His-*D*-Trp-*D*-Lys-Trp-*D*-Phe-Lys-NH_2 **3**

[N-Ac-Tyr^1,*D*-Arg^2]-GRF(1-29)-NH_2 **4**

GHRP-6 - Mechanistic Considerations - Extensive studies *in vitro* with GHRP-6 carried out by several research groups have focused on the mechanism of GHRP-6 induced GH release (83-85). Both GHRP-6 and GRF activate the hypothalamus-pituitary axis, and binding sites with affinity for both secretagogues have been identified in these tissues (86,87). However, there is a large body of evidence supporting the existence of independent secretory pathways for GHRP-6 and GRF (88-91). Studies *in vitro* with rat pituitary cells demonstrate a pronounced synergistic effect of GHRP-6 on GRF-stimulated GH release (90,91). Cells maximally stimulated by either GHRP-6 or GRF can still release additional GH when treated with the other secretagogue. The effects of specific antagonists to GHRP-6 and GRF also support a dual mechanism. The GHRP-6 specific antagonist **3** blocks GH release by GHRP-6, but has no effect on GRF-stimulated GH secretion. Conversely, the GRF-specific antagonist **4**, suppresses GH release by GRF but not GHRP-6 (88,92). The identification of a new, GRF-independent GH secretory mechanism has triggered efforts to identify an endogenous ligand for the GHRP-6 dependent pathway (88,93).

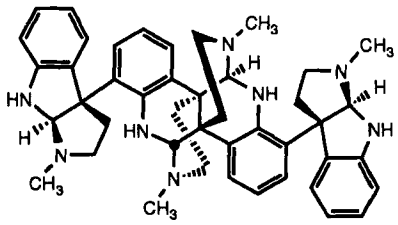
GHRP-6 - Animal Studies - A variety of studies of GHRP-6 in animal models have been undertaken to evaluate efficacy *in vivo* and to confirm the physiological relevance of the GRF-independent GH secretory pathway. Early studies in normal rats successfully demonstrated a dose-dependent GH release by *i.v.* administration of GHRP-6 (94). This study also qualitatively demonstrated that multiple dosing of GHRP-6 at regular intervals results in multiple GH responses, thus mimicking the natural pulsatile release of endogenous GH. Additional support for a second GH release mechanism *in vivo* was revealed in subsequent studies of GHRP-6 and GRF, where differences in the dose-response curves for the two secretagogues were apparent (95). Synergism of the two secretagogues *in vivo* has also been demonstrated (93,96). Oral bioavailability of GHRP-6 has been evaluated in several species and is uniformly low (<1%), presumably due to the peptidyl nature of the compound (97), although bioavailabilities greater than 30% have been achieved in a dog model by intranasal administration (98).

GHRP-6 - Clinical Evaluation - Initial clinical trials in normal men showed that GHRP-6 is safe and well-tolerated. Serum GH is elevated in a dose dependent manner; other pituitary hormones LH, FSH, TSH and ACTH remain unchanged (99). Occasional symptoms of facial flushing and sensations of warmth that have also been observed with GRF treatment are present in GHRP-6 treated subjects. Subsequent trials revealed a synergistic GH response when administered in combination with GRF, consistent with GHRP-6 acting through a non-GRF dependent mechanism (100). Desensitization of this secretory pathway has been demonstrated by continuous infusion of GHRP-6, which blunts the effect of a subsequent bolus dose (101). Oral administration of GHRP-6 in normal men shows a dose-dependent GH response, although the drug is poorly absorbed (0.3%) (102,103). Alternative routes of administration, such as intranasal, have been investigated although with limited success (96).

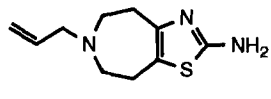
NON-PEPTIDYL SECRETAGOGUES

Several structurally diverse non-peptidyl compounds have been reported to stimulate GH release *in vitro* or *in vivo*. Many of these molecules mediate their effects indirectly through alternative mechanisms such as somatostatin antagonism or catecholaminergic, adrenergic, cholinergic and serotonergic pathways. Consequently, these compounds generally lack the high degree of specificity associated with GHRP-6 or GRF.

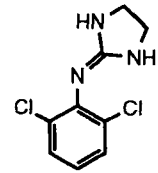
The natural product psycholeine (**5**), a pyrrolidinoindoline alkaloid, stimulates GH release *in vitro* by blocking the action of somatostatin (104). Talipexole (B-HT 920, **6**), has been evaluated clinically and shows a weak GH response by oral administration (105). The mechanism of GH release by **6** is not clear and a variety of associated CNS and cardiovascular activities limits its therapeutic potential (106). Clonidine (**7**), an α_2-adrenergic receptor agonist, releases GH by stimulating GRF release from the hypothalamus (67). The compound has been evaluated clinically in children with constitutional growth delay (107) and in patients with panic disorder (108). Hypotension, sedation and other endocrine effects have been observed in patients treated with clonidine (109).



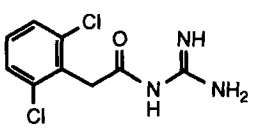
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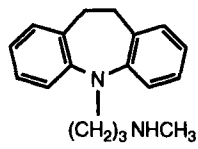
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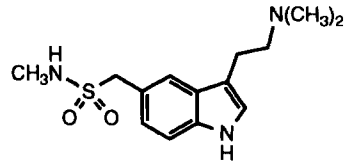
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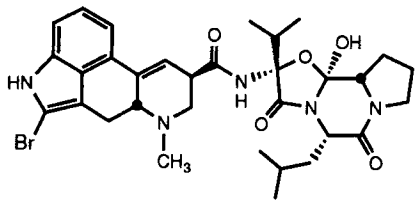


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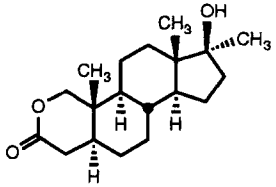
The GH releasing properties of other α_2-adrenergic receptor agonists have also been investigated clinically. Guanfacine (**8**) releases GH in a dose-dependent manner without the hypotensive or sedative effects experienced with **7** (110). The tricyclic antidepressant desipramine (**9**) stimulates GH release in normal subjects, although elevations in ACTH, PRL and cortisol are observed (111).

Sumatriptan (**10**), a serotonin 5-HT_{1D} receptor agonist used in the treatment of migraine, releases GH in man following subcutaneous administration (112). GH release is most likely effected through hypothalamic 5-HT receptors which have been implicated in pituitary hormone regulation (113). The antiparkinsonian *L*-dopa, whose GH stimulatory effects are also mediated through hypothalamic GRF (67,114), has been tested clinically in GH deficient children (115). *L*-Arginine, a relatively weak GH secretagogue, has been evaluated clinically by *i.v.* infusion in normal subjects (30 *grams* over 30 min!). Its mechanism of action is not well understood but it has been proposed that GH release is mediated through cholinergic stimulation (114).

The dopamine receptor agonist bromocriptine **11** and the steroid oxandrolone **12** have demonstrated positive growth effects clinically in children with hypopituitarism and constitutional growth delay (116,117).



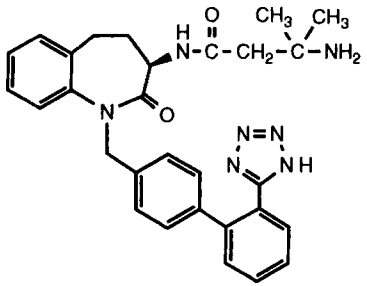
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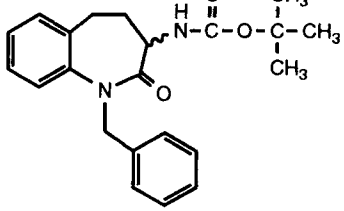
12

In contrast to GRF and GHRP-6, these compounds stimulate growth by enhancing the sensitivity of target tissues to GH and hence, neither are formally GH secretagogues. It has been proposed that oxandrolone may activate the hypothalamus-pituitary axis by sensitizing pituitary somatotrophs to GRF, thereby increasing endogenous GH release (118).

Benzolactam Secretagogues - Recently a report appeared describing the discovery of a novel class of non-peptidyl structures that stimulate GH release, the benzolactam secretagogues (119). Unlike many of the non-peptidyl compounds, benzolactam GH secretagogues are unique in that they retain the high degree of specificity demonstrated by GHRP-6. The prototype compound in this class, L-692,429 (**13**), stimulates GH release in a dose-dependent manner *in vitro*.



13



14

Studies *in vitro* indicate that **13** mediates GH release through the same GRF-independent mechanism as GHRP-6 (120). As with GHRP-6, **13** exerts a synergistic effect on GRF-mediated GH release. Cells maximally stimulated by **13** produce no additional effect when treated with GHRP-6, yet remain responsive when treated with GRF. The reciprocal antagonism demonstrated with GRF, GHRP-6 and their respective antagonists **3** and **4** is also preserved in the benzolactam series. The structurally related analog **14** blocks GH release by **13** or GHRP-6, but has no effect on GRF-stimulated GH release. The identification of this structurally distinct secretagogue that

apparently mediates GH release through the same non-GRF pathway as GHRP-6 provides additional support for the existence of a physiologically relevant endogenous ligand.

Evaluation of **13** in animal models confirms its efficacy and specificity for GH release. In beagle dogs, *i.v.* administration of **13** results in a dose-dependent increase in serum GH compared to placebo. Except for nominal increases in cortisol and ACTH, other hormones, e.g., insulin, LH, triiodothyronine (T3), thyroxine (T4) and aldosterone remain essentially unchanged (121). The time course of **13** given *i.v.* is also quite rapid; peak GH levels are attained at 15 min and return to basal levels by 75 min. Such a short acting species presents an advantage clinically by mimicking the natural pulsatile pattern of GH release.

Clinical studies with **13** show it to a be a well tolerated and relatively selective GH secretagogue. In a double-blind, placebo controlled study (122), dose-dependent elevation in serum GH is observed in healthy males upon *i.v.* administration of **13**. Symptoms of facial flushing and sensations of warmth which have been observed with GHRP-6 and GRF treatment, are also observed in a small fraction of subjects administered compound **13**. Otherwise, the drug is well tolerated with no other side effects. A similar clinical study carried out in older subjects (>65 y) showed that **13** stimulates *greater* GH secretion than GRF(1-29)-NH_2 without clinically significant adverse effects (123). In light of recent findings suggesting that GH treatment in older men reverses some of the bodily changes associated with aging (37), this last result underscores one of the many potential therapeutic utilities of growth hormone secretagogues (124).

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Chapter 20. Proteinases In Inflammation

Allen Krantz
Syntex Research Canada
2100 Syntex Court, Mississauga, Canada L5N 3X4

Introduction - The inflammatory response and its numerous mediators constitute topics of immense breadth which have been the subject of many excellent reviews bearing on the role of proteinases in tissue destruction (1-5). This report is primarily concerned with inhibition strategies published over the past two years and the current status of proteinases released by neutrophils and mononuclear phagocytes (macrophages).

Neutrophils - Tissue destruction by neutrophils in human disease has been the subject of several comprehensive reviews (6-8). Neutrophils are the first leukocytes to emerge from blood vessels in significant numbers in the typical acute inflammatory response and are frequently the predominant infiltrating cell type. When activated, neutrophils release a complex assortment of agents that can destroy normal cells and dissolve connective tissues. Approximately 50 toxins have been identified, primarily localized to the plasma membrane-associated NADPH oxidase or the intracellular granules which release a complex mix of NADPH oxidase derived oxygen metabolites and granule-based enzymes. Lysosomal cysteine proteinases with pH optima in the acid range, and three neutral serine proteinases, elastase, cathepsin G, and proteinase-3, are stored in the azurophil granules. Two metalloproteinases, collagenase and gelatinase [whose subcellular localization has been controversial (9)], are found in specific secondary and tertiary granules, respectively. In general, azurophil granules fuse with phagosomes and do not release their contents to the extracellular milieu except after stimulation by chemotactic secretagogues. Specific granules, however, fuse with the plasma membrane as they approach inflammatory sites and can release their contents extracellularly.

Macrophages - In contrast to neutrophils, macrophages are long-lived cells (many months), with important synthetic capabilities and major secretory activities, and with a remarkable potential for functional differentiation (10). Macrophages are derived from blood monocytes. Such mononuclear phagocytes express a changing profile of proteinases that vary with the developmental stage of the cells. Macrophages lack subcellular storage organelles comparable to neutrophil granules and do not contain serine proteinases, but they can internalize and secrete scavenged HLE. They do, however, synthesize lysosomal cysteine proteinases and have the capacity to secrete several metalloproteinases, including collagenases, gelatinases and stromelysins, capable of degrading the extracellular matrix. Macrophages contain large amounts of acid hydrolases mostly within secondary lysosomes, but intracellular levels of most proteins they release are relatively low and are strictly dependent upon continuous de novo synthesis. By secreting proteinases, proteinase inhibitors, and cytokines that affect proteinase production by other cells, macrophages play an important role in the degradation and remodelling of the extracellular matrix.

The Proteinase-Anti-proteinase Balance - Proteinases must perform their normal functions during homeostasis, as well as disease-related tissue destruction, in settings ostensibly dominated by high concentrations of powerful endogenous inhibitors (anti-proteinases) that can rapidly and irreversibly inhibit proteinase activity in plasma and interstitial fluids (11). A cardinal tenet guiding current research is that this delicate balance is undermined by various factors in inflammatory disease leading to uncontrolled proteolysis in line with the destructive potential of phagocytic and tissue proteinases. The classic example is the development of familial emphysema where levels of α_1-antitrypsin (α1AT), the major inhibitor of HLE in lung, are significantly reduced because of the inability of a mutant form of this protein to be secreted (12). Proposed mechanisms which may tip the balance in favour of proteolytic activity include (i) inactivation of antiproteinases by oxygen metabolites to create a microenvironment whereby released proteinases can preferentially and tightly associate with host tissues in safe haven from active endogenous inhibitors. In neutrophils a critical role in inhibitor inactivation has been assigned to HOCl (6); (ii) localization of extracellular matrix degradation to pericellular sites where tightly adhering surface-bound proteinases are concentrated and protected from inhibitors. Triggered neutrophils have the ability to create a subjacent sequestered environment from which endogenous inhibitors are excluded and released proteinases can act unmolested (13, 14); (iii) proteolytic inactivation of endogenous inhibitors by non-complexing proteinases (15); and (iv) saturation of local inhibitory activity due to excessive proteinase release from massively infiltrating inflammatory cells.

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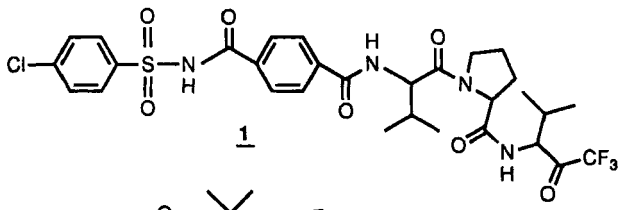
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Nevertheless, the pathogenesis of clinical disorders involving inflammation remains elusive because of the vast array of redundant mediator systems and cell-to-cell interactions, which make up a complex network tightly regulated by various amplification and feed-back mechanisms that are slowly being unravelled. Although little is known about the cellular events responsible for switching off the inflammatory response, it appears that inhibitor-enzyme complexes (16) and inactive, cleaved forms of inhibitors (17) can play a part in influencing the resynthesis of functionally active proteins required for the restoration of the protease-inhibitor balance. The recently identified serpin-enzyme complex has been shown in culture to mediate catabolism of α-antitrypsin-elastase complexes, increases in synthesis of α1AT (18), and the neutrophil chemotactic activity of α1AT-elastase complexes (19). It is becoming increasingly evident that a complex cascade of proteolytic events determines tissue breakdown and it may well be that no single enzyme is of overriding importance. The ability of cathepsin G to activate neutrophil collagenase, which in turn is capable of cleaving the reactive loops of α1ATs and rendering them inactive against HLE, has been postulated as but one example of the synergy between serine and matrix metalloproteinases, leading to unregulated destruction of extracellular matrix components (20). Such regulating mechanisms may be important in controlling proteolysis in the setting of inflammatory diseases and host defense, and offer severe challenges to medicinal chemists to identify critical, clinically relevant, proteinase targets.

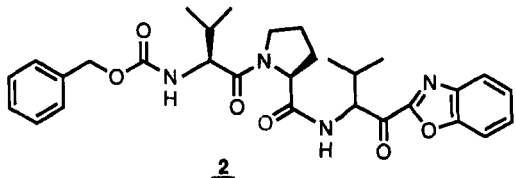
SERINE PROTEINASES

Human Leukocyte Elastase (HLE) - Neutrophil accumulation with the release of elastase has been implicated in the pathology of a host of diseases (8), where it is thought that the balance between HLE and its inhibitors is disrupted in favour of the unbound enzyme. Aerosolization of anti-elastases such as α1AT and secretory leukoprotease inhibitor in the treatment of emphysema and cystic fibrosis represent promising, important test cases for gauging the effects of localized inhibitor supplementation with minimal disturbance of normal, homeostatic functions (21-23). In a rodent animal model, proteinase-3 or cathepsin B have been successfully employed (in lieu of HLE) as elastinolytic agents to induce emphysema by intratracheal instillation (24, 25). Interstitial collagenase expression in the lungs of transgenic mice has been reported to cause pulmonary emphysema, implicating interstitial collagenase as a possible etiological agent in the disease and providing a new animal model to study the pathogenesis of emphysema (26). Severe emphysema can also be induced by repeated intratracheal instillations of E. coli lipopolysaccharide in hamster lungs which cause neutrophil influx accompanied by a release of elastase (27).

Synthetic Inhibitors - Serine proteinases have a pronounced tendency to form stable oxyanion-based tetrahedral adducts with complementary peptidyl ketones that are activated by electron-withdrawing groups. Thus ICI 200,880, 1, currently in clinical trials for the treatment of cystic fibrosis, is a selective, potent, competitive, and slow-binding inhibitor of HLE with K_i = 0.50 nM, that is also therapeutically effective in HLE-induced emphysema in hamsters. Whereas ICI 200,880 is rapidly eliminated when administered iv in dog, monkey, and hamster, and is not orally bioavailable in the latter species after a dose of 250 mg/kg, half-time retention of aerosol treatment (10 mg/ml, 60 min) in hamster was ≈10 h (28). Heterocycles with appropriate electron withdrawing properties can also activate the carbonyl of peptidyl ketones toward nucleophilic addition by the active site serine hydroxyl of HLE. The peptidyl α-ketobenzoxazole 2 has K_i=3.0 nM, and is thought to form a critical hydrogen bond between His-57 and the benzoxazole nitrogen in the hemiketal(ide) adduct, based on a closely related crystal structure determination (29). 2-(Peptidamido)-cyclobutanones are modest inhibitors of HLE with K_i ~mM, but are at least five-fold more effective than their acyclic analogs (30).



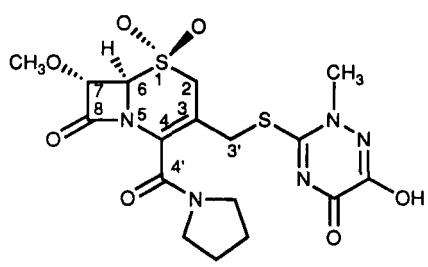
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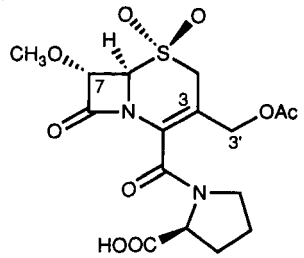
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The unique facility of serine proteinases amongst proteolytic enzymes to form acyl enzymes with various lactones and lactams, and generate stable complexes or tethered reactive intermediates, continues to guide efforts in the design of ß-lactam inhibitors of HLE (31,32). Potent time-dependent inhibitors of HLE and their structure-activity relations have been described for C-2-, 3'-, and C-7-substituted cephalosporin

t-butyl esters. HLE forms stable 1:1 covalent complexes with L-659,286 (3) and L-658,758 (4) (accompanied by low turnover) from which the 3'-leaving group has been extruded (33). A series of highly

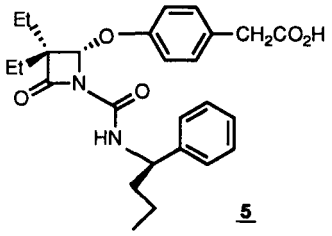


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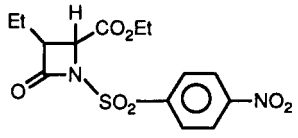


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specific, time-dependent, monocyclic ß-lactam inhibitors of HLE have also been described (34), which has led to the orally active ß-lactam inhibitor L-680,833 5 of HLE (k_{inact}/K_i = 622,000 $M^{-1}s^{-1}$). By contrast with 3 and 4, the monobactam 6 forms a stable acyl enzyme that does not lead to fragmentation (35).

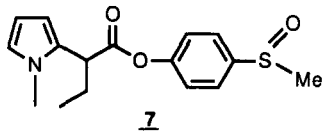


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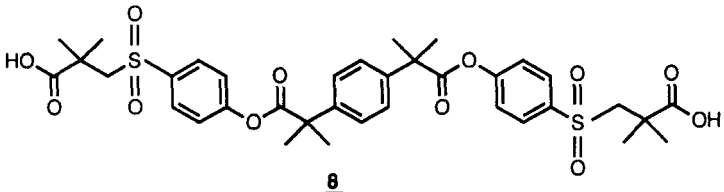


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The sulfoxide-ester CE-0266 (7) and the related sulfide and sulfone derivatives are new esters that likely form stable acyl enzymes and are alternative substrate inhibitors (36). MDL-201,404 (CE-1037, 8), a hindered phenolic ester with good aqueous stability, is a highly selective inhibitor of HLE (IC_{50} = 3 nM) which affords good protection from neutrophil-mediated damage in an extracellular matrix assay (37).



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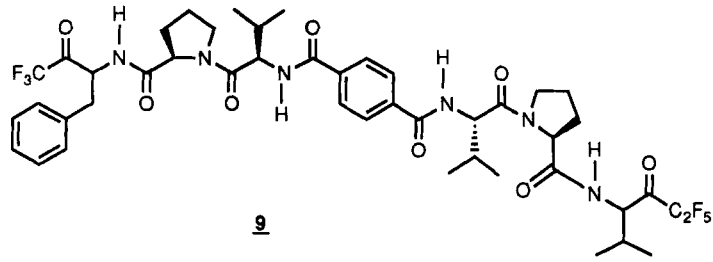
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Cathepsin G - Cathepsin G (Cat G) is a 30 kD microbicidal glycoprotein and chymotrypsin-like serine proteinase with similar physicochemical properties to elastase. By contrast with elastase, the involvement of Cat G in diseases is not as well documented, although it is often linked with HLE in neutrophil-mediated tissue destruction, but as a less potent proteolytic enzyme. A recent example involves the abrogation of neutrophil lysate-mediated cartilage proteoglycan degradation from ^{35}S-radiolabeled cartilage discs incubated with neutrophil lysate in serum-less media for 4h at 37°, which requires the inhibition of both elastase and Cat G (38).

Cat G has been shown to be a strong platelet agonist that is released by either C5a or fMLP-stimulated neutrophils and rapidly induces platelet calcium mobilization, secretion, and aggregation (39). Neutrophil-activating peptide-2, a cytokine that causes neutrophil degranulation and chemotaxis, is generated by the action of Cat G on connective tissue-activating-peptide-III and ß-thromboglobulin, as well as from Cat G bound to platelets. However, HLE degrades the precursor proteins to inactive peptides, which may signify

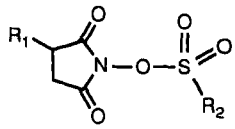
a specific role for Cat G in neutrophil activation (40).

By design, the "Janus compound" MDL 101,232 (**9**) contains dual recognition sequences capable of inhibiting HLE (K_i = 15.9 nM) and Cat G (2.2 μM) and blocking stimulated neutrophil-mediated cartilage degradation in a dose-related manner (41). 2-Alkyl-1,8-dihydroxy-anthraquinones are fully reversible, competitive inhibitors of HLE and Cat G with IC_{50} values in the micromolar range (42).



9

Proteinase-3 - Proteinase-3 (PR-3) is a recently characterized neutral serine proteinase, whose ability to degrade elastin at pH 6.5 is even greater than that of HLE, with activity at pH 7.4 that is about half that of HLE (43). PR-3 has been cloned and found to be identical to the antimicrobial protein p29b/AGP7, a serine proteinase derived from HL-60 leukaemia cells (44). It is more abundant in neutrophils than elastase, has a similar proteolytic profile and specific activity, and serves as an antigen recognized by Wegener's granulomatosis autoantibodies, a systemic vasculitis complicated by myelo-monocyte proliferation (45). The first reported small molecule inhibitors of PR-3 are 3-alkylsuccinimido-N,O-sulfonates that exhibit time-dependent inhibition (i.e., **10**, R_1=Et, R_2=CH_3 has k_{obs}/I=13,400 $M^{-1}s^{-1}$) (46). The same strategy has been used to inhibit Cat G and HLE with R_1 being aralkyl (47), or part of a fused heterocyclic ring (48).



10

Elafin inhibits elastin-FITC degradation by PR-3 with an IC_{50} = 9.5 nM (49). Known substituted isocoumarins, peptidyl phosphonates and chloromethylketones inhibit PR-3 less potently than HLE by 1-2 orders of magnitude (50).

CYSTEINE PROTEINASES

Mammalian lysosomes contain four different cysteine proteinases called cathepsin B, H, L, and S, which, as endopeptidases, have overlapping hydrolytic specificities and are involved in general intracellular turnover (51). Cathepsins B (Cat B) and H also act as a dipeptidyl carboxypeptidase and an aminopeptidase, respectively. Cathepsins L (Cat L) and S (Cat S) are the most active of the lysosomal cysteine proteinases with respect to their ability to hydrolyze azocasein, collagen, and elastin, but they are also far more tightly bound by endogenous cysteine proteinase inhibitors than is the abundant Cat B (52, 53).

Proteolytic Processing - Proteolytic processing is initiated in prelysosomal compartments and completed after arrival of the enzymes at lysosomes (54). Both Cat B and L exist as active two-chain forms which are derived from cleavage of the single chain proteins in lysosomes. In human hepatoma HepG2 cells Cat B is synthesized as an inactive 44 kD precursor and is subsequently processed to the mature 33 kD single-chain enzyme. The conversion into the 24 kD/5 kD two-chain enzyme, which occurs in the lysosome, is inhibited in cells by Z-Phe-Ala-CHN_2, which has no effect on the activation of procathepsin B to the mature enzyme (55). By contrast, the cysteine proteinase inhibitor Z-Leu-Val-Gly-CHN_2 noticeably reduced the amount of processing of procathepsin B expressed in yeast (56). The latter results parallel those with procathepsin L expressed in mouse fibroblasts, in which partially processed forms accumulate in the presence of Z-Phe-Ala-CHN_2 (57), suggesting that processing may be autocatalytic. A study of recombinant rat Cat B, expressed in yeast, shows that at least seven dissociable groups are involved in substrate binding and hydrolysis of peptidyl coumarylamide and p-nitroanilide substrates (58). Thus, there appear to be multiple steps in the post-translational processing of immature enzyme, and several loci on the mature enzyme surface that could be blocked to prevent expression of enzyme activity.

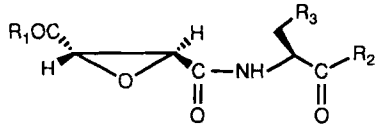
Microenvironments - The pathobiochemical significance of released lysosomal cysteine proteinases in inflammation depends on their activity outside the lysosome in the face of a high potential of endogenous cysteine proteinase inhibitors (CPI), including the kininogens, cystatins, and stefins. Another factor that

mitigates against an extracellular role for Cat B and L in tissue destruction in inflammation (52) is their acid pH optima as their activities are reported to rapidly diminish at neutral pH. The situation is not straightforward in that the activity and stability of Cat B, for example, are critically dependent on pH, temperature, and the substrates tested (59). Liver Cat B actually has greater activity at neutral pH than at acidic pH when assayed against synthetic substrates at 24°. Although under physiological conditions of pH and temperature Cat B autodegrades at a higher rate than at acidic pH, resulting in loss of activity, autodegradation can be slowed in the presence of physiologically relevant alternative protein substrates.

A role for lysosomal cysteine proteinases in the pathology of bone resorption is compatible with their ability to degrade native collagen at acid pH characteristic of sealed extracellular microenvironments enclosed by osteoclast apical and bone surfaces. From homogenates of mouse calvaria, three cysteine proteinases of bone tissues have been characterized immuno- and bio-chemically as Cat B, (pro)cathepsin L, and a Cat L-like 70 kD proteinase (60).

Synthetic Inhibitors - Despite their analogous catalytic mechanisms, cysteine proteinases are far more fastidious in their interactions with small molecules than are serine proteinases which tolerate a variety of heterocyclic substrates and inhibitors. There are few reports of non-peptidyl substrates or inhibitors of cysteine proteinases, but very potent irreversible inhibitors have been designed on the principle of appending a thiol reactive group to specific binding determinants of the target cysteine proteinase. Outside of peptidyl aldehydes, which probably have limited clinical utility because of their chemical reactivity, there are few potent reversible inhibitors of cysteine proteinases. To date, peptidyl trifluoromethyl ketones are disappointingly poor inhibitors in the high micromolar range. The most potent α-ketoesters and α-diketones reported have $K_i \approx 0.1$ μM (61).

Succinyl Epoxides - A major effort in inhibitor design has been devoted to exploring the seemingly subtle reactivity of succinyl epoxides as represented by E-64 **11** and its derivatives. The development of the prodrug loxistatin (**12**), which had been in clinical trials since 1986 as a cysteine proteinase inhibitor for treatment of muscular dystrophy, has been discontinued in Phase III (62). Although **12** had been shown to selectively act on cysteine proteinases and not affect non-proteolytic thiol enzyme activities, significant covalent binding of radiolabel to tissue proteins other than thiol proteinases is observed in rats and hamsters after oral administration of [^{14}C] loxistatin. Loxistatin causes hepatoxicity in rats, but not in hamsters (63).



11 E-64, $R_1 = OH$, $R_2 = NH(CH_2)_4NHC(=NH)NH_2$, R_3 = i-Bu

12 LOXISTATIN, $R_1 = OC_2H_5$, $R_2 = NHCH_2CH_2CH(CH_3)_2$, R_3 = i-Bu

13 CA-074, $R_1 = CH_3CH_2CH_2NH$, R_2 = N-prolyl, R_3 = s-Bu

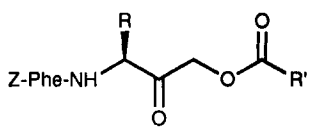
Neither **11** nor leupeptin is able to block Z-[^{125}I]iodo-Tyr-Ala-CHN_2 labelling of cysteine proteinases in intact, purified lysosomes or in various cultured cell lines, whereas **12** and specific diazomethylketones such as Phe-Ala-CHN_2, capable of penetrating membrane barriers, are effective (64). IL-1 stimulated proteoglycan release from cultured bovine nasal septum cartilage is inhibited by Z-Tyr-Ala-CHN_2, apparently without affecting signal transduction, in a dose-dependent fashion (30% at 1 μM), implicating one or more of Cat B, L, or S as mediators of IL-1-stimulated cartilage proteoglycan destruction (65). Three stereoisomers of loxistatin **12** also inhibit proteoglycan release in a dose-dependent manner (≈50% at 10 μM), but succinyl epoxides of low membrane permeability showed no inhibition at concentrations up to 100 μM (66).

Whereas **11** inactivates many cysteine proteinases, CA-074 (**13**) is a potent and selective in vivo inhibitor of Cat B (67). When injected i.p. into rats (8 mg/100 g rat), **13** selectively inactivates this enzyme as judged by post-mortem assay of B, H, and L-activities in liver (68), but does not appear to be Cat B selective in kidney (69). Since **13** does not enter cells readily, the methyl ester was used as a prodrug (1 μM, 3 h) to demonstrate selective inactivation of the intracellular Cat B of cultured human gingival fibroblasts (70).

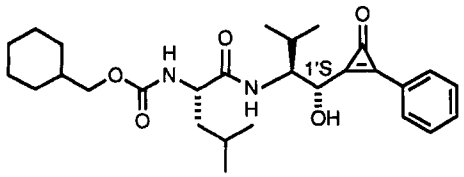
The structure of **13** was designed by computer modelling on the assumption that it binds to nonprime subsites of Cat B (71) as established for E-64. A refined 2.15 Å x-ray crystal structure of human liver Cat B, which displays a similar folding pattern and arrangement of active-site residues to that of papain, reveals that His-110 and His-111 are in a loop which occludes primed subsites and may be responsible for the dipeptidyl carboxypeptidase activity of Cat B by anchoring substrates with C-terminal carboxylates (72). Kinetic analysis of the mutant enzyme His111Glu supports this proposal in that much reduced exopeptidase activity was

observed, whereas activity with endopeptidase substrates was increased relative to wild type (73). Following this lead an alternate proposal that **13** may bind with opposite polarity from E-64 dictated by C-terminal carboxylate-His110/His-111 interactions (70, 74), with proline bound in the S_2' subsite, appears tenable.

Other Peptidyl Inhibitors - A single oral dose of 25 mg/kg of N-protected peptidyl fluoromethyl ketones such as Z-Phe-Ala-CH_2F inhibited the severity of inflammation, and cartilage and bone destruction in adjuvant induced arthritis (75). 1-Peptidyl-2-haloacetylhydrazines (halo = I, Br, Cl) are powerful inactivators of Cat B, with no detectable activity toward serine proteinases (76). Peptidyl acyloxymethyl ketones **14** represent a new design concept ("quiescent affinity labels") in the construction of inactivators of cysteine proteinases in which both the affinity and leaving group can be continuously varied in a rational way to achieve potent, chemically stable, and specific inhibitors of this class of enzyme, i.e., vs bovine spleen Cat B: Z-PheLysCH_2OCO-2,4,6-Me_3Ph has k/K=230,000 $M^{-1}s^{-1}$ (77). Peptidyl acyloxymethyl ketones are poor inhibitors of chicken smooth muscle calpain II, even with carboxylate leaving groups of low pKa and affinity groups optimized for calpain (78). The novel cyclopropenone **15** has been briefly described as a competitive inhibitor of papain (K_i = 55 nM) (79).



14



15

Cathepsin S (Cat S) - Cat S, the most recently characterized lysosomal cysteine proteinase, is similar to Cat L in its inhibitor and substrate specificity (80). Bovine spleen Cat S has been sequenced and found to be a single chain protein, in contrast to Cat B, H, and L (81). The enzyme can hydrolyze elastin over a broad range of pH values being maximally active at pH 4.5; unlike Cat B, H, and L, Cat S retains over 90% of its activity after incubation at pH 7.0 for 24h at 25° (82).

Human Cat S has been cloned and expressed independently by three groups (83-85). The cDNA of human Cat S predicts a preproenzyme consisting of 331 amino acid residues. The mature enzyme expressed in yeast has a molecular weight of 24,000 in excellent agreement with M_r of 23,979 based on the cDNA sequence (85). The reported molecular mass of 28 kD for the human alveolar macrophage enzyme expressed in COS cells is derived from a labelling experiment with an iodinated E-64 analogue (83). Human Cat S does not contain a potential glycosylation site in the mature enzyme but there appears to be one located in the proregion of the enzyme. The high stability and elastinolytic activity of the mature enzyme at neutral and slightly alkaline pH values make it a prime candidate for a role in tissue destruction.

Interleukin-1ß Convertase (ICE) - Interleukin-1ß (IL-1ß) is a major mediator of inflammation and potent bone resorptive cytokine which also mediates soft tissue destruction through the stimulation of prostaglandin production, and the induction of collagenase and other proteinases. IL-1ß is synthesized as a biologically inactive 31 kD percursor (pIL-1ß) which ICE specifically cleaves into a more active 17.5 kD form, presumably within cells, after which it is secreted (86, 87). Although there is considerable interest in ICE as a possible drug target in settings where it potentially is a unique activator of IL-1ß, some IL-1ß precursor is found outside cells where it is cleaved by other proteases. For example, human epidermal keratinocytes produce but do not process 31 kD IL-1ß, which can be cleaved by exogenous chymotrypsin and can be converted rapidly to an 18 kD biologically active species by human mast chymase. Elastase and trypsin cleave at sites which produce molecules with <2% of the biological activity of the mature cytokine (88).

The convertase, cloned and expressed by two groups (89, 90), is composed of two nonidentical subunits that are derived from a single proenzyme, and possesses a unique affinity for Asp in P_1 of its substrates. The enzyme sequence is not homologous to any known cellular cysteine proteinase, but the enzyme has been identified as a cysteine proteinase based on its inactivation by a complementary diazomethylketone, AcTyr-Val-Ala-Asp$COCHN_2$ (k_2 = $1.6x10^4$ $M^{-1}s^{-1}$), as well as by iodoacetate and disulfide reagents. Consistent with this proposal, Ac-Tyr-Val-Ala-AspCHO is a slow binding inhibitor with K_i=0.76 nM (90).

MATRIX METALLOPROTEINASES (MMPs)

At least nine distinct members of the matrixin family produced by connective tissue cells have been identified and broadly subdivided into three categories on the basis of substrate specificity: the collagenases,

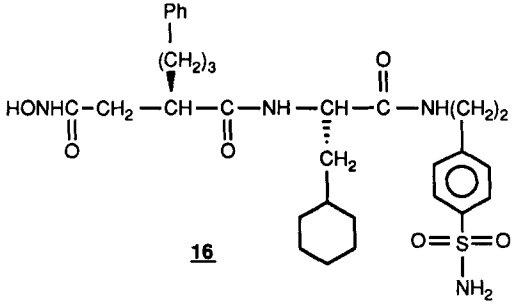
gelatinases, and stromelysins. MMPs are all Zn^{++} and Ca^{++} requiring enzymes which are secreted in inactive proforms, activated by loss of a propeptide of molecular weight ≈10,000, and then subject to inhibition by TIMP-1 or -2. These enzymes have the combined ability to degrade all the components of the extracellular matrix. Given their potential for tissue destruction, it is not surprising that regulation of MMP activity is a complex process involving control at multiple levels: gene expression (message transcription and translation) and extracellularly (activation of latent proenzymes, inhibition of enzyme activity by endogeneous inhibitors, and degradation of active enzymes), mechanisms which are under intense study. *Annual Reports in Medicinal Chemistry* has reviewed the inhibition and biochemistry of collagenase (CNase) and stromelysin (SLN) in 1990 (91). In the interim, several comprehensive reviews have appeared on MMPs and their natural inhibitors (92-97), their role in arthritides (98-100), and the status of synthetic inhibitors (101). In view of the broad, detailed coverage, this section will present only a few highlights of the past year.

Enzymes - The elusive murine macrophage metalloelastase (MME) has been cloned and identified as a new member of the MMP family with a predicted molecular weight of 53 kD for the proform. Expression of the latter in E. coli led to a 22 kD form with significant elastinolytic activity (102). The human counterpart of this enzyme is still unknown. Matrilysin, previously found only in tumors and tumor cells, is synthesized by cultured glomerular mesangial cells and expressed in vivo during an acute glomerular inflammatory process associated with IL-1 synthesis (103).

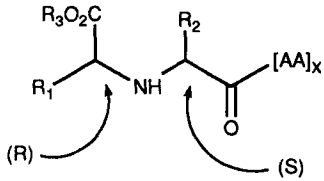
SLN concentration in joint fluid is a parameter that distinguishes diseased joints from healthy joints (104), an observation consistent with the many studies supporting a critical role for SLN in the inflammatory arthritides (98). Substrate specificity of SLN towards variants of substance P, the kinetic mechanism of SLN-catalyzed hydrolysis of such peptides, and the nature of the rate-limiting association of phosphorus containing inhibitors with SLN (ligand exchange of the inhibitor for the zinc-bound water with formation of two hydrogen bonds) have been reported (105).

Natural Inhibitors - Considerable interest attends elucidation of the properties of proenzyme-inhibitor complexes formed between progelatinases and TIMPs, which, to date, are unique among the metalloproteinases. A major proportion of the inhibitory activity of large inhibitor of metalloproteinase (LIMP), apparent M_r = 76,000, secreted in human fetal lung fibroblast culture medium, has been shown to consist of TIMP-2 in combination with 72,000-M_r progelatinase (106). The active site and the COOH-terminal tail of the 72-kD progelatinase independently and cooperatively participate in TIMP-2 binding (107). In contrast to human fibroblasts, TIMP-2 is released from macrophages free of any apparent complexed matrix metalloproteinase. By virtue of the effects of various modulatory agents, it appears that TIMP-2 biosynthesis is regulated in a distinct and completely opposite manner to that of CNase and TIMP-1 (108). In situ hybridization studies of synovial gene expression indicate that SLN and TIMP genes likely have distinct transcriptional controls which provide precise control over the local environment and matrix turnover (109).

Synthetic Inhibitors - The state of the art has evolved to enable the design of inhibitors with subnanomolar K_i's, possessing the equivalent of two amino acids and an N-terminal zinc ligand. Thus, human skin fibroblast CNase is inhibited by $HONHCOCH_2CH(i\text{-}Bu)COTrpNHMe$ (most probably the R,S-isomer) with K_i=0.4 nM/pH 6.5 (110), and (**16**) inhibits 72 kD gelatinase with K_i=0.025 nM (111). Substituted N-carboxylalkyl peptidyl derivatives (**17**) with novel C-termini have been disclosed as potent inhibitors (112).



16



17

Compared to their parent amides, the surrogate $RNal\psi[CH_2NH]AlaNH_2$ and its $\psi[CH_2SO]$ and $\psi[CH_2S]$ analogs show a significantly reduced, but still respectable, IC_{50}=40-80 nM versus pig synovial gelatinase ($R=HSCH_2CH[CH_2CH(CH_3)_2]CO$) (113). A naturally occurring family of peptide hydroxamates, the matlystatins, provide submicromolar inhibition of type IV collagenases (72 and 92 kD) and SLN (114). Tetracyclines are among the few known heterocyclic inhibitors of MMPs, with doxycycline, for example, showing IC_{50} ≈25-40 μM vs MMP-8, and inhibition of muscle proteolysis in vivo (115). Whereas a 1:1 mixture of R,S-

and S,S-HONHCOCH$_2$CHiBuCONHCHArCONHCH$_3$ (Ar=p-methoxybenzyl) inhibits release of proteoglycan from IL-1 stimulated pig articular cartilage fragments, TIMP-1 does not inhibit the release (116). Although suicide inhibitors of metalloproteinases have been reported (117, 118), these strategies have not been adapted to the inactivation of MMPs, and it is not clear that MMPs can activate such latent reactive functions.

Conclusion - Despite the many potent in vitro inhibitors, very few orally bioavailable proteinase inhibitors have been reported and evaluated in a clinical setting targeted for inflammatory diseases. Greater diversity of inhibitors of metalloproteinase and cysteine proteinases, which are currently primarily peptide-based, would be useful. There are still major gaps in our ability to design many types of inhibitors: covalent inhibitors of MMPs, and potent non-covalent inhibitors of cysteine proteinases. The need for selective, bioavailable inhibitors of specific enzymes within these subclasses is apparent.

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Chapter 21. Alzheimer's Disease: Current Therapeutic Approaches

Varghese John, Ivan Lieberburg and Eugene D. Thorsett
Athena Neurosciences, South San Francisco, California 94080

Introduction - Research on Alzheimer's disease (AD) has produced extraordinary progress in the last few years. Though the exact pathogenic mechanisms of AD remain elusive, it is known that many neurotransmitter systems are dysfunctional, neuronal calcium homeostasis is upset, processes are in motion which produce senile plaque and neurofibrillary tangles, and there is an inflammatory response associated with the disease. The lack of any proven biochemical diagnostic method for AD makes selection of therapeutic targets difficult. Nonetheless, a significant effort has been expended to develop drugs which will provide treatment for the AD population. It is the intent of this chapter to examine the status of the active AD therapeutic approaches as well as some areas which are only beginning to see medicinal chemistry activity. Our effort has been helped by the appearance of several reviews addressing some of the topics contained herein (1- 5).

NEUROTRANSMITTER REPLACEMENT

The cholinergic hypothesis of AD is based on the reported reduction of cholinergic markers such as acetylcholinesterase (AChE) and choline acetyltransferase (ChAT). According to this hypothesis medial forebrain cholinergic neurons undergo progressive retrograde degeneration accounting for some of the cognitive impairments in patients with AD. Additional support is rendered by the observed loss of cholinergic input to the cortex and hippocampus of AD brain, while muscarinic postsynaptic receptors appear to survive largely intact (1). Further, lesions to the ascending forebrain cholinergic system in animals can impair learning tasks and cholinomimetic agents reverse the process (6). The cholinergic hypothesis has provided the first rational approach to the treatment of AD and underlies the palliative treatment of AD by neurotransmitter replacement (2,3,7,8). Three approaches to enhancing cholinergic function include administration of acetylcholine precursors, inhibition of acetylcholine degradation or enhacing acetylcholine levels using acetylcholinesterase inhibitors or acetylcholine releasing agents, and directly stimulating cholinergic receptors using cholinomimetics (muscarinic agonists) (9). Other neurotransmitters known to be compromised in AD include serotonin, dopamine, and neuropeptides such as somatostatin (10). Approaches that attempt to correct these deficits form alternate neurotransmitter replacement therapies for AD (11).

Cholinergic Agents - Acetylcholine precursors, including various preparations of choline and lecithin, were the first compounds administered in AD in attempts to augment central cholinergic activity by enhancing acetylcholine levels. The precursor strategy assumes that the rate of acetylcholine release is related to the synthesis of acetylcholine, which in turn is dependent on the rate of choline uptake into the cholinergic neuron system. The results of clinical studies have so far shown little or no evidence for the efficacy of this approach (12).

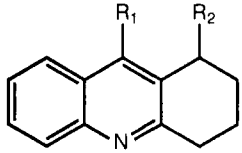
Acetylcholinesterase Inhibitors - Clinical studies on the AChE inhibitor tacrine (THA, **1**) in a small group of patients with moderate to severe AD that showed improvements in cognitive function resulted in intensified efforts to develop novel acetylcholine esterase inhibitors (13). Though subsequent clinical studies have produced mixed results (14 - 17), THA has been recommended by the FDA advisory panel for approval for AD treatment. The utility of THA in AD may be limited by its capacity to cause hepatocellular injury (18). Other derivatives of THA such as velnacrine (HP-029, **2**), a metabolite of THA which is well-tolerated in single dose studies with healthy volunteers (19), and suranacrine (HP-128, **3**) are under clinical evaluation in AD (20). The SAR studies leading to the synthesis of these compounds have been described (21).

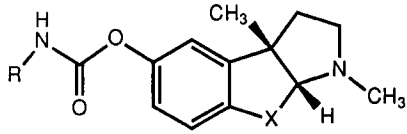
The cholinesterase inhibitor physostigmine (**4**) has been reported to have memory-enhancing effects in AD (22). Physostigmine, however, has a short half-life, variable bioavailability and a narrow therapeutic index. Heptylphysostigmine (MF-201, eptastigmine, **5**), a more lipophilic

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analogue, is reported to be less toxic than 4 while retaining its *in vitro* AChE inhibiting potency (23). The SAR of a series of 8-carbaphysostigmine analogues (6) with greater potency and reduced toxicity compared to 4 have been described (24). Galanthamine (7) is long-acting, centrally active competitive cholinesterase inhibitor which is well tolerated during long-term treatment (25) and is undergoing clinical evaluation for AD (26). In a passive avoidance test in forebrain lesioned mice the n-butyl carbamate derivative 8 was more potent and less toxic than 7 (27). The organophosphate

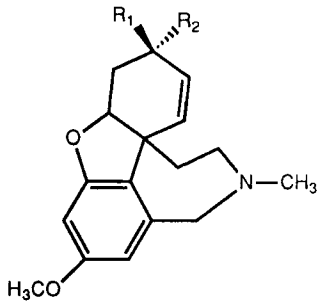


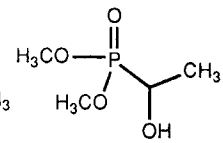


1: $R_1 = NH_2$, $R_2 = H$
2: $R_1 = NH_2$, $R_2 = OH$
3: $R_1 = NHCH_2Ph$, $R_2 = OH$

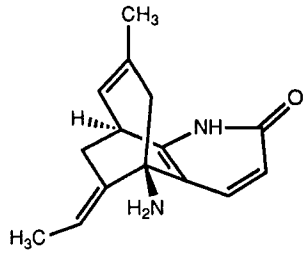
4: $R = CH_3$, $X = N\text{-}CH_3$
5: $R = n\text{-}C_7H_{15}$, $X = N\text{-}CH_3$
6: $R = CH_3$, $X = CH_2$

metrifonate (9), undergoing clinical testing for AD (28), is not itself an AChE inhibitor but is nonenzymatically transformed into an active irreversible inhibitor of the enzyme (29). Huperazine A (10) is a potent reversible AChE inhibitor with greater selectivity and potency than physostigmine and is also in clinical trial (30).





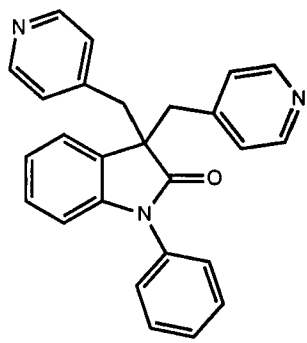
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10

7: R_1=OH, R_2= H
8: R_1 =$OCONHC_4H_9$, R_2=H

Acetylcholine Release Modulators - DUP-996 (11) enhances potassium evoked acetylcholine release from the rat cortex, hippocampus and caudate nucleus *in vitro* (31,32). Dopamine and serotonin release are also enhanced but release of glutamate, GABA, and norepinepherine are unaffected. Phase 1 clinical trials with this agent demonstrated EEG changes associated with vigilance improving properties. The EEG changes were most pronounced over temporo-occipital, temporo-frontal, parietal and frontal regions of the brain, areas reported to be the most afflicted in AD (33). 4-Aminopyridine, a potassium channel blocker, also enhances the stimulated release of acetylcholine. Clinically, some improvement in cognitive functions were reported in one study of 4-aminopyridine while another found no significant effect of the drug on cognitive performance (34).

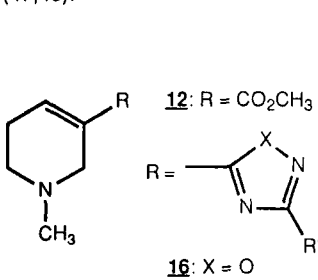


11

Muscarinic Agonists - Cholinergic agonists acting directly on muscarinic receptors may improve the cholinergic dysfunction seen in AD. Five genetically defined muscarinic receptor subtypes (m_1-m_5) have been reported to date, three of which (M_1- M_3) have been pharamacologically characterized in functional studies (35). In AD the basal forebrain muscarinic neurons that predominantly express the presynaptic M_2 receptors have been found to atrophy (36). However, the mainly postsynaptic M_1 receptors are highly concentrated in the cortex and hippocampus and their density is reported to be unaltered in AD (37). A preferential

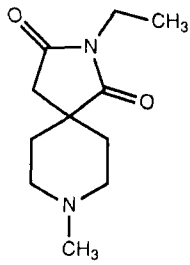
involvement of the M_1 receptors in memory has been tentatively proposed, whereas activation of central and peripheral M_2 and M_3 receptors seem more closely associated with the production of undesirable side effects (38). This suggests that M_1 selective agonists may be useful in AD treatment. Several nonselective muscarinic agonists, such as arecoline (**12**), RS-86 (**13**), oxotremorine (**14**) and pilocarpine (**15**), have been evaluated in AD patients with disappointing results (39-43).

The SAR on a series of M_1 selective arecoline analogues (**16**, **17**) which utilize 3-alkoxy/alkylthio- 1, 2, 4-thiadiazole and 1, 2, 4- oxadiazole isosteres of the arecoline ester group have been described (44). *In vivo* data indicate only minor side effects making them potential candidates for evaluation in AD. The quinuclidine analog AF102B (**18**) is a potent M_1 selective agonist that attenuates cognitive dysfunctions in animal models (45) and is under evaluation in AD (46). Agents that have dual synergistic actions on postsynaptic M_1 receptor stimulation and presynaptic M_2 receptor antagonism have been reported. The thiopilocarpine analog SDZ ENS 163 (**19**), in clinical trials for AD, and the oxotremorine analog BM-5 (**20**) fall into this category (47,48).

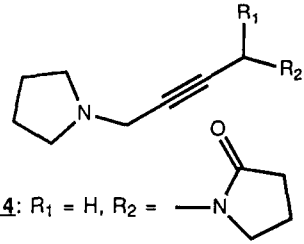


12: R = CO_2CH_3

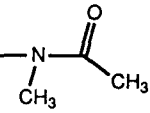
16: X = O
17: X = S



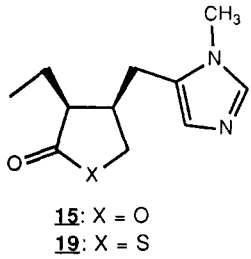
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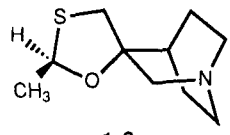
14: R_1 = H, R_2 =



20: R_1 = CH_3, R_2 =



15: X = O
19: X = S



18

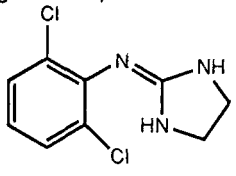
In cell culture systems, the M_1 and M_3 muscarinic receptors have been shown to be involved in the regulation of processing and secretion of Alzheimer amyloid precursor protein (APP) (49-51) which is involved in the formation of the characteristic AD senile plaques. Although these observations lend support to the hypothesis that M_1/M_3 receptor activation is an important therapeutic target, clinical evaluations of muscarinic antagonists in AD have been disappointing. This may suggest that other factors involved in the signaling pathway, such as defective coupling of the muscarinic receptor with its nucleotide-binding protein (52), may be important.

One of the potential advantages of enhancing endogenous acetylcholine release or action is that such a process would permit both muscarinic and nicotinic stimulation to occur in the AD brain. There is evidence to suggest that stimulation of central nicotinic receptors in AD patients causes increased attention and enhances general CNS stimulation. Relying on an entirely muscarinic approach to cholinergic therapy would forego any beneficial effects of nicotinic stimulation and thereby further limit the utility of such drugs.

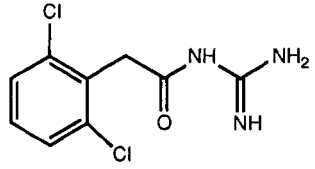
NONCHOLINERGIC AGENTS

Monoaminergic Agents - Postmortem studies of AD brain tissue show decreased levels of norepinepherine and dopamine-ß-hydroxylase (53) as well as diminished numbers of serotonin

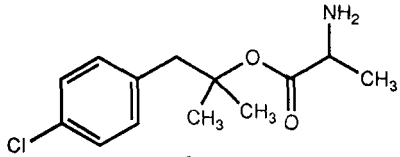
uptake and binding sites (54) while less consistent decreases in dopamine and D-2 receptor levels are seen (55). These observed abnormalities have led to alternative therapeutic strategies directed at enhancing monoaminergic neurotransmission. The α-2 adrenoreceptor agonists clonidine (**21**) and guanfacine(**22**) exhibit memory-enhancing effects in aged monkeys (56). However, clinical trials of these agents in AD patients have been unsuccessful (57,58). Serotonin uptake blockers alaproclate (**23**), zimelidine (**24**) and citalopram (**25**) have been assesed in AD and failed to produce any significant improvements in cognition (59-61).



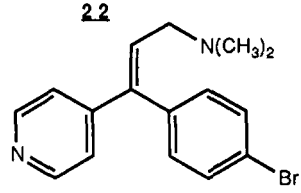
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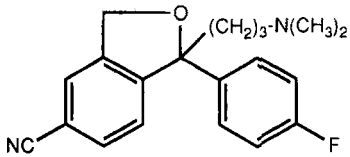
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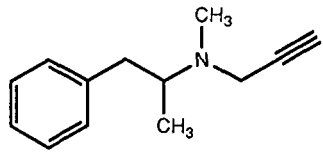
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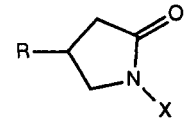


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Monoamine oxidase (MAO) inhibitors are thought to increase post synaptic monoamine transmitter availability by inactivating a monoamine catabolic pathway. Deprenyl (**26**), a potent and selective irreversible inhibitor of MAO-B, has been evaluated in AD patients. While a number of studies show significant improvements in cognitive function in treated patients, these changes may in part be due to a mild behavioral disinhibiting effect (62,63). Milacemide (n-pentylaminoacetamide) a MAO-B inhibitor and a glycine pro-drug, showed no evidence of cognitive enhancement or reduction in disease progression in AD patients (64).

Agents affecting neuropeptide systems - Somatostatin and to a lesser degree neuropeptide Y, substance P, and corticotropin releasing factor are consistently reduced in AD (65). Based on these observations neuropeptide analogs that are CNS permeable may have utility in AD treatment. Clinical studies of the cognitive effects of corticotropin (ACTH) fragments $ACTH_{1-10}$ and $ACTH_{4-9}$ have provided inconsistent results (66) possibly due to poor metabolic stability and low lipohilicity. The more lipophilic ACTH analog HOE 427 $(Met(O)_2$-Glu-His-Phe-D-Lys-Phe-$NH(CH_2)_8$-$NH_2)$ has considerably prolonged metabolic stability. Single dose studies in AD patients show slight but significant effects on attention and mood (67). In animals angiotensin II has a detrimental effect whereas the ACE inhibitor captopril has a protective effect on cognitive function (68) suggesting that captopril and possibly other ACE inhibitors as well as angiotensin II antagonists may have a role in the treatment of AD. This notion was not supported by a clinical report utilizing the ACE inhibitor ceronapril (69).

Nootropics - Piracetam (**27**) is the prototypic compound in this group whose mechanism of action remains elusive. Neither **27** or oxiracetam (**28**) had a significant effect on cognitive impairment in AD (70,71). Aniracetam (Ro 13-5057, **29**), another piracetam analog, is under clinical evaluation for the treatment of AD (70). Acetyl levocarnitine hydrochloride has been reported to have some beneficial effects in AD patients (73,74).



27: R = H, X = $-CH_2CONH_2$
28: R = OH, X = $-CH_2CONH_2$
29: R =H, X = PhCO-

Restoration of neuronal function - One of the most direct ways to restore neuronal function is to administer neurotrophins (75). Nerve growth factor (NGF), because of its action on cholinergic cells, is the one most relevant to AD. Most of the acetylcholine in the cerebral cortex and hippocampus comes from the cholinergic cells located in the ventral forebrain which degenerate early in the course of the disease and are highly responsive to NGF leading to the proposal of NGF as a potential clinical candidate for AD treatment (76). Apparently some blood-brain penetration of NGF has been acheived by conjugation of the protein to a transferrin receptor antibody (77). The conjugated NGF increased survival of both cholinergic and non-cholinergic neurons in a rat transplantation model. Molecules structurally related to NGF have been cloned and expressed including brain-derived neurotrophic factor (BDNF) and the neurotrophins NT-3, 4 and 5 (78). A study showing that the hybridization signal for BDNF, but not NGF or NT-3, is decreased in hippocampal AD tissue suggests that a decreased expression of BDNF may contribute to the progression of cell death in AD supporting the therapeutic evaluation of this neurotrophin in AD (79).

INHIBITION OF AMYLOID FORMATION AND NEUROTOXICITY

APP Cleavage - The formation and biological properties of amyloid plaque is an area of major interest (80) though considerable controversy surrounds the role of amyloid plaque in AD associated neurodegeneration (81). If amyloid derived senile plaque is a primary cause of neuronal degeneration, then inhibition of amyloid formation could provide agents with the potential to slow down or even stop the progression of AD. Senile plaque consists largely of a peptide fragment (Aß) derived from Alzheimer amyloid precursor protein (APP). The enzymatic processing of APP can proceed by any of several pathways. One secretory pathway results in the cleavage of APP in the interior of the Aß sequence and thus precludes the formation of amyloidogenic fragments. Secretase, the enzyme responsible for this cleavage, appears to exhibit specificity which depends on substrate conformation rather than sequence (82,83). An alternative lysosomal/endosomal pathway produces amyloidogenic fragments which contain the entire Aß sequence (84-87). Moreover, it is now known that a basal level of Aß production may be normal (88-90). Evidence from cell culture studies indicate that other APP cleavage pathways also exist (91), the importance of which remain to be determined.

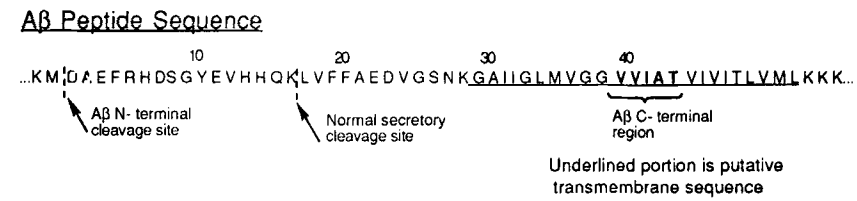


Figure: Aß sequence within APP. Aß peptide may be 39 - 42 residues in length.

Considerable evidence suggests that amyloidogenic fragments are produced in an acidic intracellular compartment, possibly via the endosomal-lysosomal pathway (83,84,92-96) although it is possible that Aß formation may be initiated by an alternative secretory mechanism (97-99). The lysosomal pathway has been implicated in other neurodegenerative diseases (100) and lysosomal proteinase distribution is known to be altered in AD (101,102). Taken together, these data suggest that intracellular proteases which operate in acidic environments may be potential therapeutic targets. Along these lines, treatment of cells with the relatively non-specific cysteine proteinase inhibitors leupeptin and E64 caused the lysosomal accumulation of APP fragments (93,94), at least one of which contained the Aß sequence (94). Bestatin, chymostatin, pepstatin A, aprotinin, soybean inhibitor, arphamenine A and B, and diprotin were without significant effect on APP processing (93). Although inhibition of lysosomal proteinases could increase the potential for amyloid formation (94), selective inhibition of lysosomal proteinases may be possible (103) such that altered proteolysis of APP fragments to non-amyloidogenic species could occur.

A partially purified calcium activated serine proteinase from AD brain was shown to be capable of making the Aß N-terminal cleavage in a model decapeptide substrate as well as the non-amyloidogenic secretory cleavage (104). The characterization profile of this enzyme is similar to cathepsin G but cathepsin G was not detectable in the tissue samples used. A similar enzyme was

isolated from monkey brain which was also capable of making the Aß N-terminal cleavage (105). It showed immunoreactivity to cathepsin G antibodies but lacked complete identity. Rat brain yielded an enzyme which exhibited macropain-like multicatalytic properties (106). In the absence of calcium the preferred cleavage site using model substrates was at the secretase site while in the presence of high calcium levels cleavage at the Aß N-terminal site was preferred.

The potential role of thrombin in AD pathogenesis (107) has received attention since this enzyme may accumulate abnormally in the AD brain. Thrombin has been shown to be present in plaques and some neurofibrillary tangles in AD brain as well as in senile plaque isolated from patients not diagnosed as AD (108). Control tissue devoid of plaque showed thrombin confined to vessel walls and residual blood plasma. Thrombin cleaves APP in the extracellular domain about 100 amino acid residues N-terminal to the beginning of Aß sequence (109). Such a cleavage would leave behind an abnormal membrane bound APP fragment whose further processing could lead to amyloidogenic fragments including Aß. Another report has shown that thrombin generates a 28kD fragment containing the Aß sequence from recombinant mouse APP (110). Clipsin, a chymotrypsin-like serine proteinase identified from rat brain, rapidly degrades APP and has been proposed as a possible Aß N-terminal cleaving enzyme though the cleavage sites have not been determined (111). Calpain I generates several fragments from APP, one of which is a 16 kD carboxy terminal fragment containing the Aß sequence (112). Proline endopeptidase is the only enzyme proposed to date as a potential Aß C-terminal cleavage enzyme (113).

Aß and Amyloid Plaque Neurotoxicity - The direct role Aß and amyloid plaque in neurotoxicity and the surrounding controversies have been extensively discussed (81). The possible role of Aß in dysfunctional calcium homeostasis in the AD brain has received attention and forms a putative link between amyloid and excitatory amino acid biochemistry. The peptides $Aß_{1-38}$ and $Aß_{25-35}$ have been shown in human neuronal cell culture to enhance calcium response to excitatory amino acid stimulation and potentiate excitatory amino acid induced neurotoxicity (114). In differentiated PC12 cells, $Aß_{25-35}$ caused a specific and dose dependent influx of calcium (115). Comparison of several Aß sequence fragments active in potentiating glutamate toxicity has led to the suggestion that the active sequence may be residues 25-28 (115), which overlap with the proposed ß-turn involving residues 26-29 in the Aß peptide (116). An alternative hypothesis suggests that Aß may be capable of forming ion channels allowing the inward migration of calcium. Thus, specific blockade of these channels may lead to agents for treatment of AD (117).

Inflammation and AD - Amyloid may be indirectly neurotoxic via the induction of an inflammatory response which could result in toxicity to neurons surrounding the inflammed plaque. It has been recognized for more than a decade that an inflammatory response is part of the overall pathology of AD (118) and has attracted considerable current interest (119). Numerous immunoreactive markers, such as complement proteins and α- antichymotrypsin (α- ACT), have been identified in association with neuritic AD plaque even though certain pathological features associated with peripheral inflammation, like edema and white blood cell infiltration, are not present. The presence of inflammatory markers associated with senile plaque has led to the proposal that AD may be an autoimmune disorder (120,121). The lack of absolute parallels between peripheral and AD-related inflammation is attributable to the differential nature of the inflammatory response in the CNS (122). Inflammation may result from injury or infection coupled with temporal changes in blood-brain barrier permeability allowing access of inflammatory proteins to the brain (123) in which case the inflammatory process might contribute materially to plaque formation (124,125). Alternatively, initiation of inflammation may not require breach of the blood-brain barrier. *In vitro* Aß itself is able to initiate the classical complement pathway in the absence of an antibody by directly binding to physiological concentrations of C1q, the first component of the C1 complex (126). When bound to the serpin-enzyme complex receptor (127), Aß mediates neutrophil chemotaxis leading to the suggestion that this interaction may be important in the local inflammatory response associated with AD (128).

It has been proposed that AD amyloidogenesis derives from an acute phase response mediated by the cytokines IL-1ß and IL-6 (129), which are produced and secreted endogenously in the brain. Both IL-1ß and IL-6 are reported to be elevated in brain and CSF of AD patients (130,131) and are implicated in the pathogenesis of AD (121,129,132). In endothelial cells IL-1ß has been shown to stimulate the expression of APP (133) as well as the secretion of APP by a protein kinase C mediated pathway (50). Inhibitors of interleukin converting enzyme (ICE), a thiol proteinase which converts the IL-1ß precursor to the active cytokine, have been designed for application to peripheral inflammation (134-136). Modulation of the effects of IL-1ß in AD may be possible through the design of CNS active inhibitors of ICE. Alternatively, inhibition of cytokine release from

microglia, which has been demonstrated using pentoxifylline and dexamethasone (137), may be a useful therapeutic approach.

It has been reported that the prevalence of AD in patients diagnosed with rheumatoid arthritis (RA) was significantly less than what would be expected in the general population (138). The result is consistent with the notion that the onset of RA occurs at a much earlier age than the onset of AD and that RA patients are generally receiving anti-inflammatory therapy which thereby offers some protection against the later onset of AD. Based on this analysis, a proposal has been put forward to examine the utility of anti-inflammatory agents as therapeutics for the treatment of AD (139). A report of the ability of intramuscularly administered deferoxamine, a drug with anti-inflammatory properties, to slow the clinical progression of AD may support this proposal (140). A recent small study has suggested that indomethacin, which exhibits CNS permeability, is able to slow the progression of AD (141). Alternatively, the underlying immunology of RA may prevent or alter the progress of AD, a possibility which remains to be addressed. Additonal epidemiological support for the notion that anti-inflammatory therapy may be useful in AD are the observations that patients with leprosy who have been treated with the anti-inflammatory drug dapsone develop less AD (142) and have fewer senile plaques at autopsy than controls (143). Again, the underlying contributions of leprosy itself and the leprosy therapy to these observations need to be dissected to determine the predictive variable.

Neurofibrillary tangles - Along with senile plaques, neurofibrillary tangles (NFT) are characteristic neuropathological lesions of AD (144,145). The exact relationship between senile plaques and NFT remains unknown. However, from a temporal standpoint, NFT occur later in the disease process than do amyloid plaques. A major protein component of NFT is tau, a family of microtubule associated proteins which are important for maintainance of the neuronal cytoskeleton. NFT associated tau is excessively phosphorylated (146,147) which may result from neuronal calcium dysregulation (148). Prior to incorporation in NFT, the abnormal phosphorylation of tau may lead to microtubule destabilization and cytoskeletal disruption resulting in impaired neuronal function and survival (145). In a rat model, disruption of the neuronal cytoskeleton leads to abnormal axonal accumulation of APP due to inhibition of axonal transport processes (149). A number of kinases have been proposed as possible phosphorylation enzymes for tau (144,145,150-155) suggesting that specific kinase inhibitors may be useful medicinal targets (4).

Conclusions - We have tried to assess the current state of the significant effort directed toward the discovery of therapies for AD. Research directed toward neurotransmitter based therapies has enjoyed a high profile in medicinal chemistry and provided numerous compounds for clinical trials. Unfortunately, the results have been mixed at best. Furthermore, the increasing numbers of neurotransmitter deficits associated with AD diminishes the specificity of any one of them as a therapeutic target (139). However, this has been offset by the significant progress made in understanding the molecular basis of the disease. The intense interest in APP processing pathways and the biological effects of the Aß peptide have opened new areas for medicinal chemistry including intervention in Aß formation and APP metabolic pathways or prevention of the potential lethal effects of Aß including calcium dysregulation and inflammation.

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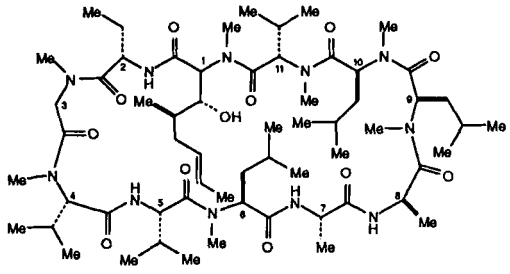
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Chapter 22. Immunophilins and Immunosuppressive Drug Action

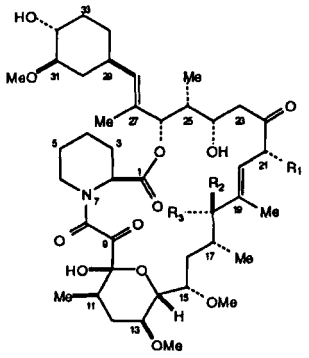
David M. Armistead and Matthew W. Harding
Vertex Pharmaceuticals Incorporated
Cambridge, Massachusetts 02139-4211

The cyclic peptide Cyclosporin A (CsA) (1) and macrolide antibiotic FK-506 (2) are potent immunosuppressants and reviews on the biological activity of these agents have appeared (1,2). CsA is currently the principle agent used for immunosuppressive therapy in solid organ transplantation and results of ongoing clinical trials with FK-506 suggest a therapeutic index that may surpass CsA (3). Rapamycin (3), a macrolide related structurally to FK-506, is also a potent immunosuppressant (4), but its clinical efficacy in human trials has yet to be established.

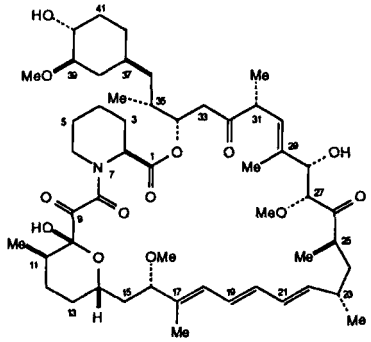
Numerous studies have examined the cellular and molecular mechanism for CsA, FK-506 and rapamycin mediated immunosuppression. Despite their dissimilar structures, both CsA and FK-506 inhibit Ca^{+2} dependent signal transduction events essential for transcription of early T cell activation genes, including IL-2, IL-3, IL-4 and GM-CSF (1,2,5). Results of several studies indicate that CsA and FK-506 block the activation of transcription factors (NFAT, NFkB, NFIL-2A, NFIL-2B, Oct-1, AP-1 and AP-3), their nuclear translocation, or association with cytokine gene enhancer elements (6,7). Conversely, rapamycin does not inhibit Ca^{+2} dependent early T cell activation events, but blocks Ca^{+2} independent steps in growth factor [IL-2 , IL-4, erythropoietin (EPO) and fibroblast growth factor (FGF)] stimulated proliferation of T cells, fibroblasts, endothelial cells, hepatocytes, and probably other cell types (8,9). Progress in understanding the biochemical and biophysical basis for the action of these drugs is the subject of this review.



1



2: R_1 = Allyl; R_2 = R_3 = H
4: R_1 = Ethyl; R_2 = R_3 = H
5: R_1 = Ethyl; R_2 = H; R_3 = OH
6: R_1 = Ethyl; R_2 = OH; R_3 = H



3

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THE IMMUNOPHILIN FAMILY OF IMMUNOSUPPRESSIVE DRUG RECEPTORS

Initial investigations by Handschumacher and colleagues identified cyclophilin (CyP-A) as a specific cytosolic receptor for CsA (10). Subsequent studies identified a common receptor for FK-506 and rapamycin, FK-506 binding protein, FKBP12, specifying the 11,820 M_r drug receptor protein (11,12). Both CyP-A and FKBP12 are peptidyl prolyl *cis-trans* isomerase (PPIase), enzymes that catalyze the *cis* to *trans* isomerization of peptidyl-prolyl amide bonds in peptide and protein substrates (13,14). PPIases are implicated in assisting *de novo* folding of nascent proteins during translation (15). Additional members of the mammalian CyP and FKBP families have been identified, and Table 1 summarizes their M_r, drug affinity, PPIase substrate preference and catalytic efficiency, and cellular localization.

Table 1: Properties of Mammalian FK-506, Rapamycin, and Cyclosporin A Binding Proteins

Receptor Protein	M_r	KI	PPIase Specificity	Catalytic Efficiency k_{cat}/K_m	Cellular Localization	Ref
FKBP12	11,820	0.4 nM FK-506 0.2 nM Rapa	ALPF	4.3×10^6	cytosol	11,12, 16
FKBP13	13,200	38 nM 3.6 nM	ND	ND	Endoplasmic reticulum	17
FKBP25	25,235	>160 nM 0.9 nM	AAPF	8×10^5	cytosol (nucleus?)	18
FKBP52	51,810	10 nM 8 nM	ALPF	3.9×10^5	nucleus & cytosol	19,20, 21
Cyclophilin A	17,737	2 nM CsA	AAPF	1×10^7	cytosol	13,22
Cyclophilin B	23,500	84 nM	AAPF	6.3×10^6	Endoplasmic reticulum	23
Cyclophilin C	22,795	ND	AAPF	ND	cytosol	24
Cyclophilin D	19,981	ND	ND	ND	mitochondria	25
Cyclophilin-40	~40,000	~ 300 nM	AAPF	1.9×10^6	cytosol	26

Both the FKBP and cyclophilin families share several features: a core sequence of approximately 90 residues which is conserved (~ 60% homology) in the sequences of FKBP12, -13, -25 and -52 (see reference 19 for sequence comparisons) and flanked by divergent N- and C-terminal extensions. Conserved residues form the FK-506 and rapamycin binding pocket [residues Y26, D37, R42, Q53, E54, I56, W59, Y82, H87 and I91 (27,28)] and the presumed PPIase active site in the proteins. Regions with sequence divergence may serve as signals for membrane anchoring, as recognition sites for physiologic partner proteins or for targeting of FKBP's to cellular compartments. FKBP52, which associates with the 90 kDa heat shock protein (hsp90) as part of non-transformed steroid receptor complexes, is a representative example for structural divergence yielding functional variation (29). A similar region of conserved sequence homology (corresponding to residues 18-132 within the CyP-A sequence) is observed within the cyclophilin family (30), also flanked by divergent N- or C-terminal extensions.

Other immunophilin family members have been identified from a wide variety of species, including *Saccharomyces*, *Neurospora*, *Drosophila*, *E. coli*, and others (30,31). FKBP12 and FKBP13 have been characterized from several species and more than 20 cyclophilins have been described to date, with the pace of immunophilin protein discovery exceeding definition of their

physiologic cellular functions. The *Drosophilia melanogaster* ninaA cyclophilin is an integral membrane protein anchored by its hydrophobic C-terminal into the membrane of the endoplasmic reticulum (ER) and in transport vesicles of photoreceptor cells. The core domain of the protein extends into the lumen of the ER where it is involved in the folding or transport of the major rhodopsin Rh1 protein (32,33). Mutations in ninaA result in improper processing or translocation of Rh1 and defective visual transduction (34,35). These observations from ninaA and FKBP52 suggest a spectrum of other roles for immunophilins in cellular physiology, independent of their association with immunusuppressive drugs.

PPIase INHIBITION IS INSUFFICIENT FOR IMMUNOSUPPRESSIVE DRUG ACTION

Early investigations examined the direct role of immunophilins in immunosuppression to address if PPIase inhibition was the sole requisite property for drug action. Several studies examined the correlation between immunophilin affinity, immunosuppressive activity, or nephrotoxicity (36-38). Consensus states that immunophilin binding is 'necessary, but not sufficient' for immunosuppression. Potent immunosuppression by the weak PPIase inhibitor [MeBm$_2$t}-1-CsA , and lack of immunosuppression by the potent PPIase inhibitors MeAla6-CsA, and the FK-506 analog (*S*)-18-hydroxyascomycin (5) clearly dissociate PPIase inhibition from immunosuppression (see Table 2, ref. 38). Further studies demonstrated that FK-506 and rapamycin are reciprocal antagonists *in vitro*. Drug concentrations 10-100 fold higher than the effective 'agonist' overcome excess drug binding sites resulting in displacement of drug effectively from its biological effector (39). Similar antagonism of CsA is observed with MeAla6-CsA (37). 506BD, a ligand designed to contain a FKBP binding domain common to FK-506 and rapamycin, but lacking other putative 'functional' elements of these drugs, was prepared to extend this concept (40). 506BD is a potent inhibitor of FKBP12 PPIase activity, is devoid of immunosuppressive activity, and is an antagonist of both FK-506 and rapamycin (40). (*S*)-18-Hydroxyascomycin has similar dual antagonist activity for FK-506 and rapamycin (38). These findings are evidence that immunophilin-drug complexes function as the biological effectors of immunosuppression, mediated by interaction of the complex with tertiary target proteins.

Table 2: Immunophilin Affinity, Calcineurin Phosphatase Inhibition, and Immunosuppressive Activity of FK-506 and CsA Analogs

Analog	Immunophilin Affinity (K_i)	Immuno-suppression (IC_{50})	Calcineurin Inhibition Enzyme Assay (K_i)	Calcineurin Inhibition Cell Lysate Assay (IC_{50})
CsA	2 nM	40 nM	110 nM	8 nM
[MeBm2t]1-CsA	170	80	50	55
D-Dap8-CsA	2	450	>3000	>1000
D-Dab8-CsA	2	150	>2000	>1000
Me-Ala6-CsA	9	>1000	>2000	ND
FK-506	0.4	0.5	4	1
FK520 (21-Ethyl)	5	1	90	ND
FK523 (21-Methyl)	0.8	2	230	ND
15-O-Desmethyl-FK520	15	>1000	>5000	ND
(*S*)-18-Hydroxyascomycin	0.7	>25,000	>2000	ND
506BD	5	>10,000	>1000	ND

IMMUNOPHILIN-DRUG COMPLEXES BLOCK PHOSPHATASE OR KINASE ACTIVITY

Utilization of immobilized FKBP12/FK-506, or CyP-C/CsA complexes as affinity probes for isolation of putative 'effector' proteins proved to be an effective strategy, and aided identification of calcineurin, a Ca^{+2}/calmodulin dependent protein phosphatase, as a common target for FKBP12/FK-506 and CyP-C/CsA complexes (41, 42). Both classes of immunophilin/drug complexes (FKBP12/FK-506, CyP-A/CsA, CyP-B/CsA, and CyP-C/CsA) inhibit the phosphatase activity of calcineurin in *in vitro* enzyme assays, or in cellular lysates (see Table 2; and references 43-47). Rapamycin however, does not participate in formation of a ternary complex with FKBP12 and calcineurin. Consistent with the cellular antagonism studies described above, rapamycin, 506BD and (*S*)-18-hydroxyascomycin are antagonists of FKBP12/FK-506 inhibition of calcineurin phosphatase activity. Evidence from FK-506 and CsA analog studies (Table 2) shows a strong correlation between immunophilin/analog inhibition of calcineurin, and immunosuppression. Also, overexpression of calcineurin in T cells renders cells more resistant to CsA or FK-506 and augments NFAT and NFIL-2A activation of the IL-2 promoter (48,49). These results are strong evidence that calcineurin is a pivotal enzyme in the T cell receptor signal transduction cascade.

Studies of early events in IL-2 receptor linked signal transduction have identified a possible target for the FKBP12/rapamycin complex. IL-2 binding to the IL-2 receptor stimulates the phosphorylation of ribosomal protein S6 by either the p70 S6 kinase, or a member of the p90 (rsk) family of S6 kinases (50-52). Recent investigations have shown that rapamycin inhibits the activation of p70 S6 kinase (not the p90 S6 class or MAP kinase), resulting in diminished phosphorylation of the S6 protein (50-52). Consistent with cellular and biochemical antagonism studies, FK-506 is a potent antagonist of rapamycin inhibition of p70 S6 kinase activation (9). However, p70 S6 kinase is not the tertiary target protein for the FKBP12/rapamycin complex. Rather, the FKBP12/rapamycin complex blocks the activation of p70 S6 kinase, possibly by acting on an upstream kinase or phosphatase that links immediate IL-2 (or other growth factors) receptor occupation with p70 S6 activation, and subsequent steps in cell proliferation.

MOLECULAR ANALYSIS OF IMMUNOPHILINS AND IMMUNOPHILIN COMPLEXES

Several published reports have recently appeared in the literature detailing studies directed toward understanding the molecular elements involved in the inhibition of calcineurin by immunophilin/drug complexes. Biophysical analysis by x-ray crystallographic and NMR techniques have provided molecular maps of unbound and ligated FKBP12 and CyP and analysis of the differences between free and bound conformations of the ligands and their binding proteins has started to provide insight into the nature of the recognition site for calcineurin (53). In addition, structure-activity relationships of both ligand analogs (38,43) and immunophilin mutants (54-56) have identified specific residues in FKBP12 that are necessary for calcineurin mediated immunosuppression. This section will highlight these studies.

Biophysical Studies of FKBP12 The solution structures of free bovine (57) and human (27) FKBP12 have been solved by multidimensional NMR methods. The bovine and human proteins differ only by three conservative substitutions and solution conformations of these two are essentially identical. The regular secondary structure of FKBP12 is characterized by a hydrophobic binding core that contains six of the protein's nine aromatic side chains. This core is enclosed by a five-stranded antiparallel β sheet with a right-handed twist, a short α helix and two flexible loops. The two loops, at residues 39-45 and 82-94, were poorly defined by the NMR experiments.

The addition of FK-506 (**2**) to human recombinant FKBP12 yields a one to one complex and the atomic structure of this species has been solved to 1.7 Å resolution by single-crystal x-ray diffraction (28). The ligand binds tightly in the hydrophobic binding pocket with the pipecolinyl, pyranose and cyclohexyl rings, the C-10 ketone and the C24-C26 region forming van der Waals contacts with hydrophobic residues of the enzyme. Five hydrogen bonds between FK-506 and FKBP12 further stabilize the complex. Approximately 50 % of the drug is encompassed by the protein with a portion of the cyclohexyl ring (C26-C34) and the region of the macrocycle that contains the allyl group (C18-C23) exposed to solvent.

The conformation of FKBP12 is not greatly affected upon binding to ligand. The hydrophobic binding core, which is in direct contact with the drug, the β sheet and the helix undergo only small perturbations. The two flexible loops, however, are now well defined in the x-ray structure and are localized adjacent to the binding pocket. As will be discussed below, mutation studies of these two loops have shown their importance in the recognition of calcineurin (54,55).

In contrast, the bound conformation of FK-506 is markedly different from that observed for the free ligand. The uncomplexed ligand exists as a 2 :1 mixture of *cis* : *trans* amide rotamers when observed by NMR in organic solvents (58,59) but crystallizes exclusively as the *cis* isomer (60). The x-ray and NMR structures of the complex, however, shows the drug bound as the *trans* rotamer which significantly changes the shape of the molecule (28,62). There is a dramatic shift in the orientation of the pyranose ring which imparts a higher degree of hydrophobicity to the binding core of the ligand and the C-15 methoxy moves to an orientation which allows this group to come into close contact with the solvent surface. This substituent has been shown to be critical for calcineurin inhibition (53,54). It should be noted that the structure of unbound FK-506 has not been studied in aqueous solution due to solubility problems and the possibility exists that the aqueous conformation of the free ligand may more closely resemble the bound orientation. This type of conformational change in aqueous solution has recently been suggested for CsA (61).

The solution structure of ^{13}C-labeled FK-506 bound to hrFKBP12 has been determined via heteronuclear and isotope filtered NMR experiments (62). This study identified a solution conformation that is nearly identical to that observed in the crystal of the complex with the exception of the allyl group where multiple conformations were observed. In the crystal, the high degree of order seen in the allyl region is thought to be a consequence of crystal contacts. A similar report on the solution conformation of ^{13}C-labeled ascomycin (**4**) complexed with hrFKBP12 supports these studies (63).

An isotope-filtered NMR solution structure determination of two unlabeled, non-immunosuppressive analogs of ascomycin bound to [U-^{13}C,^{15}N]hrFKBP12 has provided additional detail to the understanding of the molecular interactions necessary for calcineurin inhibition (64). The two semisynthetic derivatives, (*S*)- and (*R*)-18-hydroxyascomycin (**5** and **6**), were isolated from a selenium oxide oxidation of **4**. The *S*- diastereomer (**5**) has been shown to bind to FKBP12 with high affinity and to be a potent inhibitor (K_i = 0.7 nM) of the PPIase activity of this protein (38). This compound, however, does not inhibit the proliferation of T cells activated with ionomycin plus PMA nor does it inhibit the phosphatase activity of calcineurin at concentrations up to 2 μM. Similar properties have been indicated for the *R*-isomer **6** (64). Comparison of the solution structure of either of these two derivatives with that of the parent ascomycin shows striking similarities (rms deviation for the *R*-diastereomer is 0.36 Å while that for the *S*-isomer is 0.49 Å upon superposition of the common heavy atoms of these two derivatives with the parent compound) indicating that the conformation of the bound ligand is unperturbed by the addition of a hydroxyl group at position 18 of the macrocycle. The 18-hydroxyl substituent resides on a solvent exposed region of the ascomycin scaffold and appears to disrupt the ligated FKBP12 from interacting with calcineurin. It has been noted that the protein backbone of FKBP12 adjacent to the bound ligand is hydrophobic in nature and that the introduction of a hydrophilic residue in this region may prohibit a hydrophobic interaction with calcineurin (64). The solution structure of the FKBP12/ascomycin complex by three- and four-dimensional NMR techniques has also been solved and this structure was reported to be very similar to the x-ray structure of the FKBP12/FK-506 complex (65).

The atomic structure of rapamycin (**3**) bound to hrFKBP12 has been solved by x-ray crystallography and, in contrast to FK-506, the conformation of rapamycin changes little upon binding (66). The unbound crystal structure of rapamycin has been reported (67) and this structure is nearly identical to that observed for the bound conformations of both rapamycin and FK-506. The primary difference in the bound structures of rapamycin and FK-506 is the positioning of the cyclohexyl residue. This group is influenced in the rapamycin structure by a hydrogen bond from C-40 to Gln-53, an interaction absent in the FK-506 complex. Rapamycin binds in the same hydrophobic cleft as FK-506 and a comparison of the two ligated proteins indicates that only the main chain conformation of residues 31-34 of the FKBP12/rapamycin complex differ from the FKBP12/FK-506 structure previously discussed. As has been described above, the FKBP12/rapamycin complex is not an inhibitor of the phosphatase activity of calcineurin and the immunosuppressive mode of action of rapamycin is mechanistically distinct from that of FK-506 and CsA.

Mutagenesis Studies of FKBP12 Site directed mutagenesis of surface residues proximal to the active site of FKBP12 have been performed in order to further delineate the molecular basis for the inhibition of calcineurin by the FKBP12/FK-506 complex (54, 55). These investigations were guided by the biophysical studies of FKBP12 and by the comparison of its primary sequence with other members of the FKBP family. In the first report, forty single point surface mutations were generated and the results of these variations were analyzed by comparison of the specific mutants as PPIases and in their ability to inhibit the enzymatic activity of calcineurin (54). Thirty of these

mutants had little effect on the ability of the complexed variant to inhibit calcineurin indicating that, in general, mutagenesis of the surface residues does not cause a gross change in protein conformation or enzymatic activity. However, four specific point mutations, R42I, R42K, H87V and D37V, resulted in up to a 175-fold decrease in the affinity of the FKBP-variant/FK-506 complex for calcineurin compared to wild type FKBP12. In this subset, only the D37V mutant affects FK-506 affinity or the specific activity of the PPIase catalysis. A R42K/H87V double mutant had 550-fold lower affinity for calcineurin compared to wild type suggesting a direct role for the R42 and H87 residues in calcineurin recognition. A later study produced two FKBP12/FKBP13 chimeras (55). The sequences of these two FKBP family members differ most in residues 40-44 (40s loop) and residues 84-91 (80s loop) and the purpose of the study was to splice the loop sequences of FKBP13 into FKBP12 to determine if the sequence identity in either or both of these two regions is responsible for the functional distinction of these proteins as calcineurin inhibitors. Of the two chimeras produced, only the one with the FKBP13 sequence substitution in the 40s loop maintains its ability to inhibit calcineurin when complexed with FK-506. The chimeric protein with the 80s FKBP13 loop showed 73-fold lower affinity for calcineurin compared to wild type FKBP12 implicating the role of this region in calcineurin binding. It is interesting to note that single point mutations in the 40s loop of FKBP12, R42I, R42K (54) or R42A, or R42Q (55), lead to variants of less affinity for calcineurin while replacement of the entire 40s loop which contains a R42Q mutation reduces calcineurin affinity. These data suggest that either of the 40s loops found in FKBP12 or FKBP13 can recognize and inhibit calcineurin. To test the role of the 80s loop sequence of FKBP12 in calcineurin inhibition, this sequence was grafted into FKBP13 producing a chimeric protein that was 55-fold more potent a calcineurin inhibitor compared to wild type FKBP13 (56). A P89G/K90I double FKBP13 mutant was equipotent with wild type FKBP12 in calcineurin inhibition.

The biophysical studies of ligated and unligated FKBP12 and the site directed mutagenesis work on FKBP12 have had dramatic input into the understanding of the molecular nature of the FKBP12/FK-506/calcineurin interface. Structure-activity relationships of ligand analogs (38,43) and protein analogs (54-56) indicate that regions of both FK-506 and adjacent FKBP12 residues form the calcineurin recognition element. The design of new immunosuppressive agents that exert their effect through this pathway will undoubtedly be influenced by this information.

Biophysical Studies of Cyclophilin (CyP)-The structure of unligated CyP has been obtained from an x-ray crystallographic study of free CyP (68) and an x-ray determination of CyP bound to a model tetrapeptide substrate (69). In addition, a global fold has been determined for hrCyP using both amino acid specific and uniform ^{15}N labeling strategies (70). The spherical protein consists of an eight-stranded antiparallel β barrel that exists as two perpendicular four-stranded ß sheets. The ß barrel encloses a hydrophobic core and is capped at each end by two short α helices. NMR analysis of a complex formed between CyP and a water-soluble CsA derivative shows that, unlike other ß-proteins, this hydrophobic core does not comprise the binding site for CsA (1) (69). Although the spectral data for free and bound CyP indicate that, in analogy with FKBP12, no change in secondary structure occurs on binding, chemical shift differences were observed that indicate that the CsA-binding pocket is a deep groove localized between the face of the ß barrel and a loop formed between residues Thr-116 and Gly-130. This region also incorporates the tetrapeptide binding site indicating that the enzymatic active site for CyP is located within the CsA binding domain. There is no obvious structural similarity between unligated CyP and unligated FKBP12.

The solution conformation of uniformly ^{13}C-labeled CsA (71,72) and uniformly ^{15}N-labeled CsA (72) complexed to hrCyP using multidimensional NMR techniques has been reported and the two methods have been compared (73). As is the case with FK-506, the conformation of unbound CsA as determined by single crystal x-ray analysis (74) and in organic solution by NMR (75) is dramatically different from that observed for the complexed ligand with a rms deviation between the backbone of the two structures of 2.5 Å (72). The secondary structure of CsA in the absence of CyP consists of a two stranded twisted ß sheet joined by type II' ß turn which is stabilized by three transannular hydrogen bonds and an irregular loop which is stabilized by a hydrogen bond between the NH of D-Ala8 and the CO of MeLeu6. Six of the seven N-methyl groups are oriented to the outside of the macrocycle and all of the amide residues are *trans* except for the *cis* amide bond which exists between MeLeu9 and MeLeu10. In contrast, the complexed structure of CsA determined in these NMR experiments, shows no elements of secondary structure with no intramolecular hydrogen bonds. The macrocyclic ring has in essence turned inside-out with five of the seven N-methyl groups now pointing to the interior of the ring and the four N-H residues pointing out. All of the amide residues are now in a *trans* conformation as had been previously described for [U-^{13}C-MeLeu^{9,10}]CsA in complex with CyP (76). As in the case of FK-506, the

comparison here is for unbound CsA determined in an aprotic solvent. A recent study suggests that the aqueous solution structure of CsA is probably more similar to the bound conformation of the drug (61).

Both of these NMR data sets have been used in docking simulations with the x-ray structure of unbound CyP (68) to produce models of the CyP/CsA complex (77,78). Intermolecular NOE's were observed for CsA residues 3,2,1,11,19 and 9 indicating close contacts (less than 4 Å) of these residues with CyP and the chemical shift differences observed for these amino acids on binding indicate the interaction of these residues with the hydrophobic aliphatic and aromatic residues of CyP. CsA residues 4-8 show no NOE interactions with CyP and are proposed to be solvent exposed and presumably constitute a portion of the CyP/CsA calcineurin recognition site. These models offer a good explanation for the inhibitory and immunosuppressive properties of a variety of cyclosporin analogs. Two other models based on these data have also been proposed (7,79).

The solution conformation of unlabeled CsA and MeAla6-CsA (a nonimmunosuppressive analog) complexed to fully deuterated CyP has been reported (80) and the results bear striking resemblance to the analogous investigations of inactive FK-506 analogs described above (63). In this study only the ligands had observable proton signals and the bound conformations were assigned using standard two dimensional NMR techniques. The solution conformation of CsA obtained by this method agrees with the structures from the previously discussed NMR studies. As in the case of the conformational studies of the ascomycin derivatives, the bound solution structure of the nonimmunosuppressive analog MeAla6-CsA is nearly identical to that observed for complexed CsA with a rms deviation of 0.54 Å. The primary conformational difference between these two compounds lies in the single amino acid replacement at position 6 which has been shown to be in the region of the ligand that is exposed to solvent and not involved in binding to CyP. The implication that perturbation of this residue disrupts interaction of the immunophilin/ligand complex with calcineurin is supported by position 6 analogs of CsA that bind tightly to CsA but are poor immunosuppressants (81).

The solution structure of the CsA/cyclophilin complex was recently obtained by heteronuclear three-dimensional NMR spectroscopy techniques using unlabeled CsA complexed with [U-^{13}C,^{15}N]CyP, [U-^{15}N]CyP, [U-^{13}C]CyP and fractionally ^{13}C-labeled CyP (82). In agreement with prior reports, the conformation of CyP was shown to change little upon binding while the CsA structure adopted an orientation identical to that described by the earlier NMR studies (71,72,80). The complex was shown to be stabilized by several hydrophobic interactions between the protein and the binding domain of the ligand (residues 9-11 and 1-3) as well as three intermolecular hydrogen bonds. No hydrogen bond was observed between the MeBmt1 OH and Asn 102 which had been proposed in earlier models to explain the importance of the MeBmt OH in binding to CyP and for immunosuppressive activity (78,79). The inhibitor binds to the same region of CyP as a tetrapeptide substrate but in an opposite N- to C- terminal orientation. CsA residues 5-8 do not interact with the protein and were determined to be solvent exposed. They were postulated to be a portion of the calcineurin binding site as is supported by structure-activity relationships of CsA analogs substituted in these positions.

The atomic structure of CsA complexed with CyP has also recently been solved to 2.8 Å resolution using x-ray crystallographic techniques (83). The tetragonal crystal used in this study is composed of a pentamer of 1:1 CyP/CsA complexes which pack in pairs to form a decameric asymmetric unit. The average rms deviation between the five CyP molecules is 0.59 Å and the average rms deviation is 0.26 Å for the CsA molecules. The conformation of CsA is similar to that determined in the previously described studies with the exception of the identification of a hydrogen bond between the MeBmt1 OH and the CO of MeLeu4 which was also detected in the NMR solution of the complex (82). As has been previously determined, the binding core of CsA is buried in a hydrophobic cleft of CyP with roughly 55% of the ligand left exposed to solvent. Four direct intermolecular hydrogen bonds between the enzyme and the inhibitor were observed.

CONCLUSIONS

The understanding of the biochemical and biophysical basis for the immunosuppressive efficacy of the cyclosporins and macrolides has progressed dramatically since the identification of the immunophilins as the major binding proteins for these naturally occurring immunosuppressants and their derivatives. The discovery of the role of calcineurin in the cascade of events leading to immunomodulation by CsA and FK-506 further delineates the biochemical pathway that is

interrupted by these natural products. A structural analysis of the interactions of this protein with immunophilin/ligand complexes should provide additional information for drug design. It is anticipated that the detailed molecular understanding of these interactions will enable the design of novel agents with improved therapeutic parameters and will also stimulate structural investigations into other biochemical pathways.

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Chapter 23. New Potential Therapies for the Treatment of Atherosclerosis

Scott D. Larsen and Charles H. Spilman
The Upjohn Company, Kalamazoo, MI 49001

Introduction - Atherosclerotic cardiovascular diseases are a major cause of morbidity and mortality. Aggresive therapy to decrease low density lipoprotein cholesterol (LDL-C) inhibits the progression of coronary atherosclerotic lesions, promotes regression of existing lesions, and results in a decline in the number of coronary events (1,2). Epidemiologic data consistently support the conclusion that coronary artery disease is also inversely related to high density lipoprotein cholesterol (HDL-C) levels (3,4). Therefore, the level of total or LDL-C must be carefully evaluated in the face of low HDL-C levels (5).

Following a brief overview of current therapies for hypercholesterolemia (HC), this chapter will highlight recent advances in the development of new approaches to the treatment of HC and atherosclerosis. Primary emphasis will be placed on programs which are at or near the stage of clinical development, but a final section will also focus on current basic research that suggests new ways to confront coronary artery disease.

OVERVIEW OF CURRENT THERAPIES

Approved treatments for HC have changed little since the last survey of this area in Annual Reports (6) and have been the subject of recent monographs (7,8). This section will deal only briefly with existing therapies to put current research on new approaches in an appropriate context.

Bile Acid Sequesterants - Patients who prove refractory to dietary intervention are usually treated initially with bile acid sequesterants. New developments in this area related to improvements in potency and palatibility have been reviewed (9). Highlights include tablet formulation (10), resins containing amide functionality to augment the binding capacity of the ammonium groups (11), and water-soluble polymers of reduced molecular weight (12). An alternative approach is inhibition of bile acid transporters with novel tethered cholic acid derivatives (13).

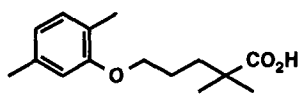
Nicotinates - Nicotinic acid was first prescribed for the treatment of HC over 25 years ago and continues to be utilized despite its well-known side effects. Efforts to dissect the untoward side effects have involved both prodrugs and timed-release formulations (7,14). The only new analog with significant advantages is acipimox (5-methylpyrazinecarboxylic acid-4-oxide) which is effective at lower doses than nicotinic acid and encumbered with fewer side effects (15).

Probucol - Although originally developed as an antihypercholesterolemic (16), probucol's effects on serum C are modest and it is of interest today primarily as a result of its antioxidant properties. Evidence implicates oxidatively-modified LDL as a factor in the generation of the aortic fatty streak, from which atherosclerotic lesions develop (*vide infra*). Probucol has been demonstrated to retard the progression of atherosclerosis in Watanabe rabbits (17), and clinical studies have indicated that it protects human LDL from *ex vivo* peroxidation and reduces plasma levels of lipid peroxides (18-20).

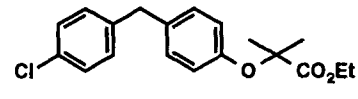
Fibrates - The fibric acids are employed primarily for the treatment of combined hypertriglyceridemia and HC (7). Although no clear structural definition of this class of compounds appears to exist, the most potent possess an α,α-dimethylacetic acid moiety attached by a 0-7 carbon spacer to a phenoxy moiety which may be further substituted. Gemfibrozil (**1**) enjoys a market edge over other fibrates and maintains nearly 30% of the total US hypolipidemic market. More active fibrates have been developed, most notably beclobrate (**2**), which is about nine times as potent as gemfibrozil (21).

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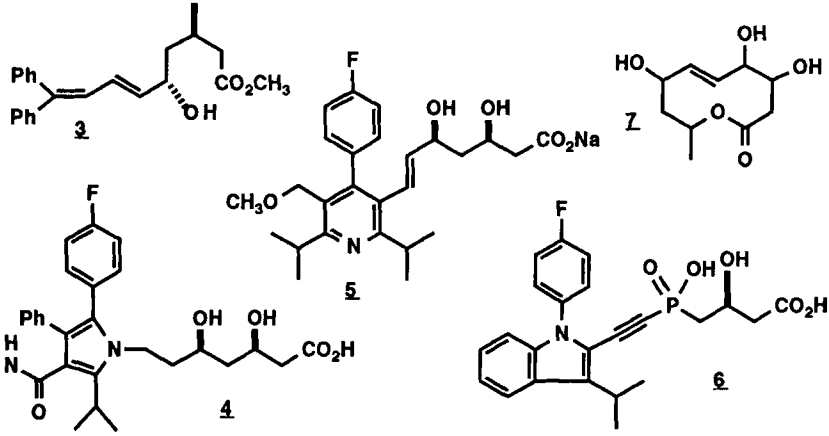
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2

HMG-CoA Reductase (HR) Inhibitors - The standard by which all new therapies for primary HC are now judged is represented by the inhibitors of the rate-limiting step in endogenous C biosynthesis. Virtually all of the members of this class of compounds are 3,5-dihydroxypentanoic acids (or the corresponding δ-lactones) further substituted with a lipophilic anchor that enhances binding to the enzyme. The number of analogs of the original lead in this area, compactin, has grown explosively in the last five years, and their development and SAR have been exhaustively reviewed (22,23). Clinical efficacy of the currently marketed inhibitors (lovastatin, simvastatin, pravastatin) is impressive: average reductions of serum LDL-C levels in patients with heterozygous familial HC range from 32 - 41% at doses of only 40 mg/day (24). Although chronic therapy has yet to reveal the serious side effects which were anticipated to arise from inhibition of C biosynthesis in the periphery (25, 26), current research is strongly focused on developing inhibitors that are selective for the liver, the primary source of endogenous C production (27). New entities of interest with regard to increased potency or hepatic selectivity relative to lovastatin are: CP-83101 (3) (28), CI-981 (4) (29), and Bay-w-6228 (5) (30). SQ-33600 (6) is particularly noteworthy in that the 5-hydroxy moiety of the pentanoic acid fragment has been successfully replaced by a phosphinyl oxygen (31).

The macrocyclic lactone (7) is reported to inhibit HR with an IC_{50} of 0.1 μM in HepG2 cells, is roughly equipotent with lovastatin at reducing HC in rats and represents an unusual departure from the standard mevalonate-mimic structure (32). The observation that an HMG-CoA thioether sulfoxide binds with greater affinity to HR than the physiological substrate thioester is intriguing and could be of importance in the design of future analogs (33).

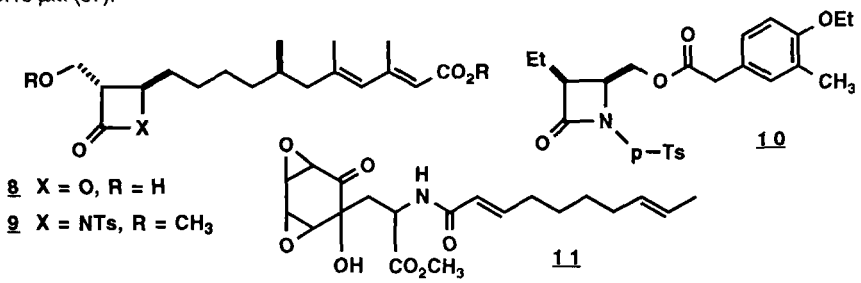


NEW THERAPIES UNDER DEVELOPMENT

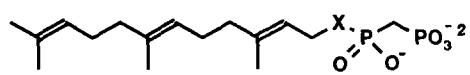
The undisputed success of current therapy for HC, particularly the HR inhibitors, has not deterred interest in discovering novel methods to reduce serum C or retard the progression of atherosclerosis. This section will detail those approaches that have identified discreet chemical leads possessing well-established biological activity. The majority of work has focused on inhibiting C biosynthesis at points other than HR and on altering the absorption of dietary C.

HMG-CoA Synthase (HS) Inhibitors - HS catalyzes the C biosynthetic step just prior to the reduction of HMG-CoA. SAR work on the original lead L-659,699 (8) established that manipulation of the lipophilic side chain (34) is detrimental to activity, but that the β-lactone can be

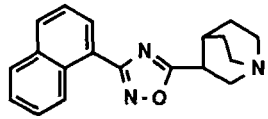
substituted with an N-p-tosyl β-lactam (9, 35). Subsequent analog work identified (10) as one of the most potent of these tosyl lactams with an IC_{50} of 2.1 nM (36). A second class of inhibitors was recently isolated from fermentation broths. The most active component (11) had a modest IC_{50} of 0.18 μM (37).

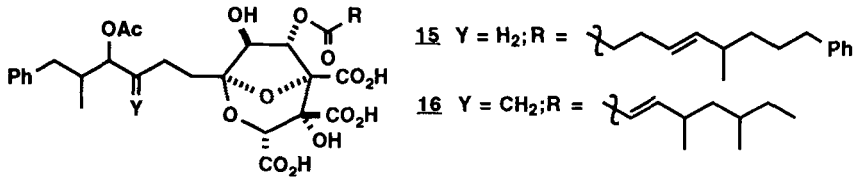


Squalene Synthase Inhibitors - The first three steps in the C biosynthetic cascade that are committed solely to sterol synthesis involve the conversion of farnesyl pyrophosphate (FPP) to lanosterol. The enzymes responsible are squalene synthase (SS), squalene epoxidase (SE), and 2,3-oxidosqualene-lanosterol cyclase (SLC). Inhibition of these enzymes is considered potentially advantageous over HR inhibition in that the biosyntheses of other isoprenoids essential for cell growth (e.g. ubiquinone) are not affected. SAR studies within a series of stable FPP analogs resulted in the potent competitive SS inhibitor (12) (IC_{50} = 0.05 μM), which proved to be two orders of magnitude more active than the closely-related compound (13), illustrating the importance of the ether oxygen for efficient binding to the enzyme (38). Azabicyclo[2.2.2]octane (14) inhibits rat liver microsomal SS with an IC_{50} of 11 nM in the presence of pyrophosphate (39), but it is part of a series of compounds also reported to be 5-HT_3 antagonists, casting doubt on its clinical potential. A group of fungal metabolites was recently isolated that exhibits extremely potent inhibition of SS (40). Due to their virtually simultaneous discovery by two different groups, they are known collectively as either zaragozic acids or squalestatins. Zaragozic acid C (15) has an IC_{50} of 9 nM against rat liver SS (41). Administration of the closely related analog squalestatin 1 (16) subcutaneously to mice results in inhibition of hepatic C synthesis with an ED_{50} of 0.2 mg/kg (42). Furthermore, (16) is orally active in marmosets at reducing serum C (43). Cycloheptylaminomethylene-1,1-biphosphonic acid (YM 175) displays significant non-competitive inhibition of rat liver SS and reduces hepatic C biosynthesis when administered subcutaneously to rats (44).

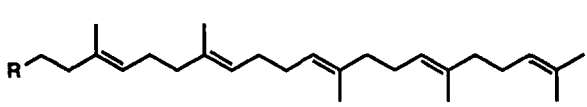


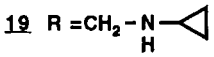
12 X = OCH_2
13 X = CH_2CH_2



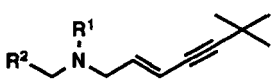


Squalene Epoxidase Inhibitors - Inhibitors of mammalian SE fall into two general classes: analogs of the physiological substrate squalene and substituted allyl amines. Trinorsqualene alcohol (TNSA, 17) was one of the first squalene mimics to effectively inhibit SE (IC_{50} = 4 μM)(45). The 1,1-difluorosqualene (18) is orally active in mice, as indicated by dose-dependent reductions in hepatic C synthesis (46). Roughly equivalent *in vitro* potency is seen with cyclopropylamine (19) against rat hepatic SE (47). SAR studies with TNSA analogs confirm that the length and degree of unsaturation of the polyene is important for activity (48).

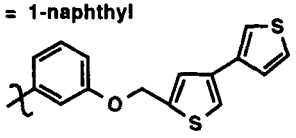


17 R = CH_2OH
18 R = $CH=CF_2$
19 R = CH_2-N (H) 

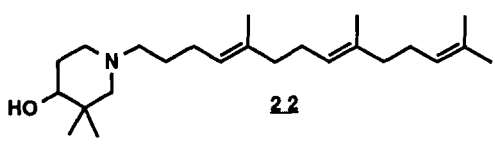
Significantly more robust inhibitors evolved from SAR work on the potent fungal SE inhibitor terbinafine (20), which itself is only a weak inhibitor of mammalian SE (49). A dramatic reversal of this selectivity was achieved with NB-598 (21), possessing an IC_{50} of 0.75 nM against HepG2 SE (50) with no accompanying antifungal activity. Furthermore, NB-598 effects reductions in serum C and increases in serum squalene in dogs following oral administration (51). Analogs with improved *in vitro* potency have been reported (52).



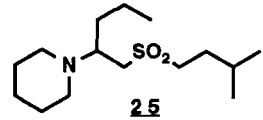
20 $R^1 = CH_3$, R^2 = 1-naphthyl

21 R^1 = Et, R^2 = 

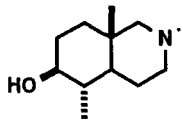
Oxidosqualene Cyclase Inhibitors - The physiological substrate for SLC, 2,3-oxidosqualene (OS), has served as the template for a number of synthetic inhibitors. 2,3:18,19-Dioxidosqualene inhibits rat liver SLC non-competitively with an IC_{50} of 0.11 μM (53). The location of the second epoxide is important, as indicated by the approximately 1000-fold lower potency of 2,3:22,23-dioxidosqualene in the same assay. Simple addition of a methylidene substituent at the 29-position of 2,3-oxidosqualene affords a potent (IC_{50} = 0.5 μM) inhibitor of pig liver SLC (54). Of particular mechanistic interest was the observation that this inhibition was *irreversible*, providing evidence that carbocationic character at C-19 was developing during interaction with the SLC and subsequently being trapped by a biological nucleophile. The hypothesis that OS actually cyclizes via discreet intermediates with carbocationic character (55) has inspired the synthesis of amino analogs of both squalene and partially cyclized squalene intermediates with the expectation that the charged ammonium group is capable of functioning as a stable bioisotere for a carbocation. A recent acyclic example is N-methyl-10-aza-10,11-dihydro-2,3-oxidosqualene with an IC_{50} against hepatic SLC of 5 μM (56). Roughly equivalent activity is observed with the monocyclic derivative (22), the most potent of a series of cyclic analogs (55). Saturation of the side chain of (22) totally attenuates its inhibitory activity. Evidently even an amide can suffice as a carbocationic mimic; azadecalin (23) inhibits hepatic SLC with an IC_{50} of 0.7 μM, comparable to the older bicyclic amine (24) (55,57). The piperidine sulfone (25) maintains significant hepatic SLC inhibition (IC_{50} = 5 μM) within a structure markedly simpler than the squalene analogs (58).



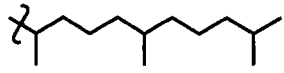
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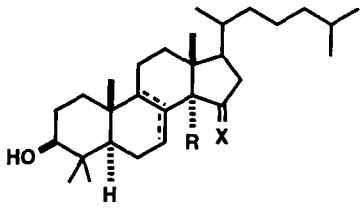


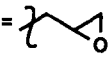
23 R = $C(CH_2)_{10}CH_3$ (O)

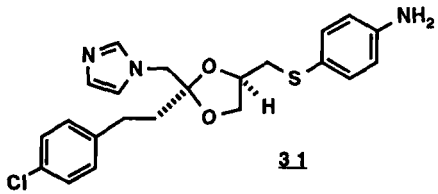
24 R = 

Lanosterol 14α-Demethylase (LDM) Inhibitors - Inhibition of the P-450 enzyme LDM has the potential to attenuate cholesterol biosynthesis by a dual mechanism: direct blockage of the conversion of lanosterol to C and indirect inhibition via a putative feedback mechanism whereby the accumulation of oxylanosterol intermediates (26 and 27) regulates the expression of HR (59). The ethynyl lanosterol (28) is reported to function as an irreversible inhibitor of rat liver LDM (60) while epoxide (29) is a competitive inhibitor with a K_i of 0.6 μM (61). Lanosterols bearing

substitution at the 15-carbon have been targeted due to their expected resistance to elimination of the 14-methyl group as formic acid, the final transformation induced by LDM. Oxime (30) is a modest inhibitor of LDM (IC_{50} = 55 μM), but is an effective oral hypocholesterolemic in hamsters (62). Reports of clinical hypocholesterolemic activity (63) with ketoconazole, an imidazole LDM inhibitor marketed as an antifungal, have fostered continued interest in the development of more potent analogs. A recent example is imidazole (31) which has an IC_{50} against hepatic LDM that is less than one-tenth that of ketoconazole and is at least three times as potent as ketoconazole at reducing serum C in hamsters (64).

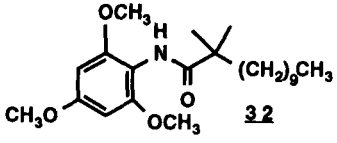


26 R = CH_2OH, Δ - 8, X = H_2
27 R = CHO, Δ - 8, X = H_2
28 R = CH-C≡CH, Δ - 8, X = H_2
29 R =  , Δ - 7, X = H_2
30 R = CH_3, Δ - 8, X = NOH

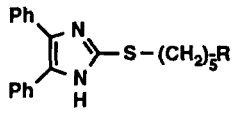


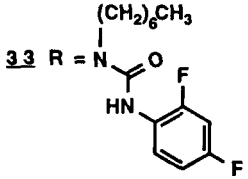
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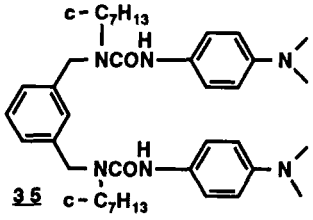
AcylCoA:Cholesterol Acyltransferase (ACAT) Inhibitors - It is now widely accepted that ACAT, an enzyme responsible for esterifying C with fatty acids, plays a key role in both the absorption of dietary C and in the accumulation of C within arterial tissue, two processes that are intimately involved in the atherogenic process (65). Research aimed at developing regulators of ACAT has been intense despite their disappointing clinical performance to date (66). The discovery and development of recent leads through 1992 have been comprehensively reviewed (67). A simple description of an ACAT pharmacophore is precluded by the diversity of reported inhibitors, but the majority to date have been lipophilic derivatives of amides, ureas, and imidazoles. Some of the most effective members of these classes which are currently in clinical development are CI-976 (32) (68), DuP-128 (33) (69), RP-70676 (34) (70), YM17E (35) (71), and 447C88 (36) (72).



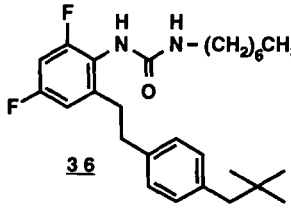
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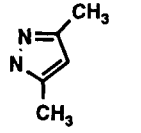
33 R = 



35

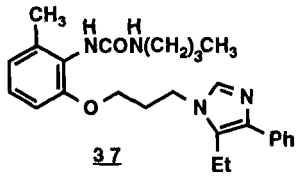


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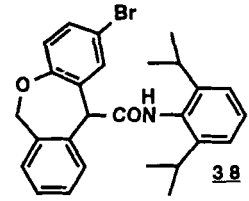
34 R = 

Attenuation of dietary C absorption has been readily achieved with a variety of ACAT inhibitors, regardless of their systemic bioavailability, as a result of inhibition of intestinal ACAT. Current research is strongly focused on developing inhibitors that act directly at the artery to determine if lesion development can be retarded. Although reports of arterial ACAT inhibition have appeared, the experimental results are often clouded by a concomitant reduction in serum C, which alone is sufficient to alter arterial ACAT activity and lesion progression. Cyclandelate (3,3,5-trimethyl

cyclohexyl hydroxyphenylacetate) was the first ACAT inhibitor to reduce aortic C without significant changes in serum C (73). Similarly, CI-976 (**32**) decreases foam cell formation and C ester content in mechanically-induced arterial lesions in micropigs at a dose which does not alter plasma LDL-C or HDL-C levels (74). Treatment of rabbits with pre-established HC with E5324 (**37**) reduced aortic arch ACAT activity by 50% without decreasing plasma C levels below controls (75). KF-17828 (**38**) is reported to accelerate the regression of HC in previously C-fed hamsters, implying a systemic effect more profound than simple withdrawal of dietary C (76).



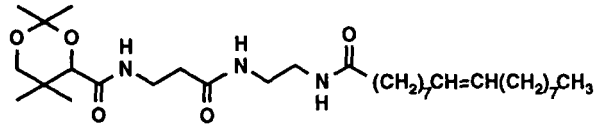
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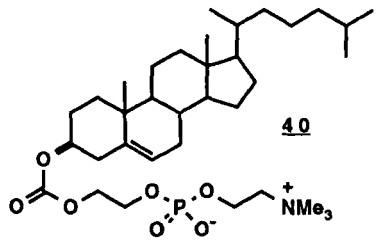
38

Several new compounds are of interest for their structural novelty. 2,3,5-Trimethyl-4-hydroxyphenyl oleamide is a modest ACAT inhibitor (IC_{50} = 0.25 μM), but is effective at reducing serum C in hypercholesterolemic hamsters and protects LDL from oxidation (77). Pantothenic acid derivative (**39**), clearly designed to mimic the preferred ACAT substrate oleoyl-CoA, is a modest ACAT inhibitor (IC_{50} = 1.2 μM) (78). Replacement of the oleyl portion with a urea affords F-1394 with dramatically improved potency (IC_{50} = 12 nM) against rat liver ACAT (79). A recently isolated fungal metabolite, the cyclic hexapeptide beauvericin (IC_{50} = 3 μM), is one of the most potent ACAT inhibitors of microbial origin (80).

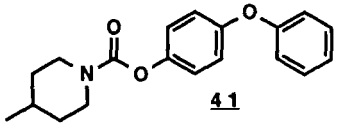
39



Cholesterol Lipid Absorption Inhibition - Beyond intestinal ACAT inhibition, the level of effort directed towards inhibiting C or fat absorption has been relatively minor. A recent patent reports significant reductions in both TC and LDL-C in human subjects given high-viscosity water-soluble cellulose ethers, presumably as a result of inhibition of C absorption (81). β-Tigogenin cellobioside (CP-88818) lowers plasma LDL/VLDL C in a variety of both C-fed and chow-fed animals (82). The mechanism of action is reported to involve sequestration of both dietary and endogenous biliary C. Phosphorylcholine derivatives of C (e.g. **40**) effect reductions in dietary C absorption in mice of 60-80% by an undetermined mechanism (83). Cholesterol ester hydrolase (CEH) inhibition remains of some interest, despite controversy over its overall impact on C absorption (65). WAY-121898 (**41**) reportedly inhibits CEH with an IC_{50} of 0.2 μM and reduces absorption of a single dose of C in normal-fed rats with an ED_{50} of 10 mg/kg (84). The pancreatic lipase inhibitor tetrahydrolipstatin (Ro-18-0647) is an inhibitor of C oleate absorption, but not free C, in rats (85), and continues to be the subject of analog work (86).



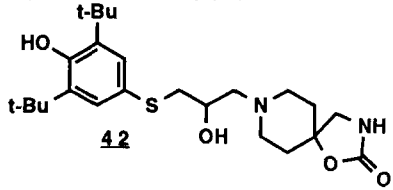
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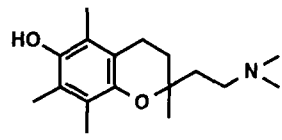
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Antioxidants - It is now widely accepted that oxidative modification of LDL accentuates its atherogenicity by a variety of mechanisms and that agents acting to inhibit this process may retard the atherosclerotic process (87,88). This view is supported by studies with the antioxidants vitamin E (89) and probucol (*vide supra*) which clearly demonstrate a variety of antiatherogenic

effects, and by epidemiological data linking coronary heart disease with susceptibility of LDL to oxidation (90). The search for more potent antioxidants has primarily uncovered compounds similar to probucol or vitamin E, i.e. lipophilic hindered phenols. Noteworthy are the spirocycle (**42**), which was 10-100 times as potent as probucol against LDL oxidation by Cu^{+2} or endothelial cells (91) and tocopherol analog (**43**) which reduced serum C in a dose-dependent manner in mice (92). The desmethyl probucol analog MDL-29311 is slightly more effective than probucol at inhibiting LDL oxidation *ex vivo* and retarding lesion progression in Watanabe rabbits, perhaps due to superior bioavailability (93).

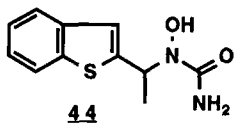


42

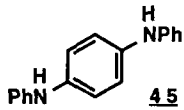


43

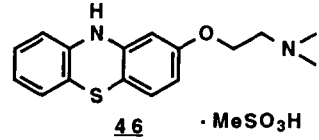
Non-phenolic antioxidants have also been reported. The hydroxyurea (**44**) has an IC_{50} of 0.5 μM for inhibition of endothelial cell-induced LDL oxidation (94). A 71% reduction in arterial lesion area in C-fed rabbits was observed upon administration of N,N'-diphenyl-phenylenediamine (**45**), accompanied by significant protection of the LDL from oxidation (95). Phenothiazine (**46**) was approximately ten times more potent than probucol at blocking Cu^{+2}-catalyzed oxidation of LDL (96).



44



45



46 · $MeSO_3H$

POTENTIAL NEW THERAPEUTIC APPROACHES

Several genes that are important in lipoprotein metabolism provide the opportunity for new therapeutic approaches. For example, a great deal is already known about the molecular mechanisms that regulate HR transcription (97) and degradation (98-100). It is now quite easy to develop systems to discover drugs that specifically regulate gene transcription. Such an assay has been developed for the HR gene (101), and assays could be developed for any gene of interest in the C biosynthetic pathway. Posttranscriptional down-regulation of HR has been reported with 15α-fluoro-lanost-7-en-3β-ol (102), 24(S), 25-oxidolanosterol (103), and γ-tocotrienol (104). Investigations to study the regulation of expression of HS (105), SS (106), and SE (107,108) are also underway. LDL receptors play a crital role in the catabolism of C, and a great deal is known about the molecular regulation of LDL receptor expression (109-111). Therapeutic agents that stimulate the production of LDL receptors, and thus lower serum LDL-C, could be discovered in an assay employing the transcriptional regulatory elements from the LDL receptor gene (112). Transgenic mice that overexpress the LDL receptor have lower plasma C concentrations than normal mice (113), and do not develop HC when fed a high-fat diet (114). One caveat to this approach is the suggestion that up-regulation of LDL receptors in peripheral tissues could lead to foam cell formation and contribute to atherogenesis (115). Apolipoprotein E (apo E) is a ligand for the LDL receptor and an important component of several lipoproteins (116,117). Intravenous administration of apo E to rabbits causes a reduction in plasma C (118). Severe HC and atherosclerosis develop rapidly in mice that lack apo E (119,120). Transgenic mice that overexpress apo E have low lipoprotein C levels and are protected from developing HC when fed a high-C diet (121). These experimental observations suggest that increasing the expression of apo E could provide protection against atherosclerosis. The first step in the conversion of C to bile acids is catalyzed by cholesterol 7α-hydroxylase (C7H)(122). Drugs that stimulate C7H activity would be expected to increase C catabolism. Stimulating expression of the C7H gene might lead to a disproportionate increase in the expression of the LDL receptor (123), and thus a large decrease in LDL-C. Drugs that lower lipoprotein(a), a lipoprotein particle that has been shown to have a positive association with atherosclerosis (124-126), could be important for the prevention of atherosclerosis in those patients who have elevated levels.

Since the incidence of coronary heart disease is inversely correlated with HDL-C levels, drugs

that increase HDL could be very important agents for the prevention of atherosclerosis. Transgenic mice that express high levels of human apo A-I, the major protein component of HDL, have elevated HDL-C and do not develop fatty streak lesions when fed a high-fat diet (127). Another potential mechanism to increase HDL is to inhibit the activity of cholesteryl ester transfer protein (CETP), a plasma protein responsible for the transfer of cholesteryl ester from HDL to other lipoproteins (128). Humans who have a CETP deficiency have very high HDL levels (129). Non-insulin-dependent diabetes mellitus (NIDDM) is associated with hypertriglyceridemia, decreased HDL-C and atherosclerosis (130,131). Patients with NIDDM are insulin resistant; thus, drugs such as pioglitazone that improve the sensitivity to insulin (132) may have a favorable impact on HDL-C levels .

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SECTION V. TOPICS IN BIOLOGY

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Chapter 24. RGD-Containing Proteins and Bone

Pamela Gehron Robey, Ph.D.
Bone Research Branch
National Institute of Dental Research
National Institutes of Health, Bethesda, MD 20892

Introduction - Bone is a remarkably specialized connective tissue that plays a number of critical functions including support and protection of other organ systems including marrow, a site for attachment of the muscular system, and a reservoir for calcium and phosphate. This review will present a brief description of the structure of bone, the organic and inorganic components of the extracellular matrix, and the cells that are intimately involved in bone destruction (osteoclasts) and bone formation (osteoblasts). Recent advances in the characterization of bone matrix proteins point to the involvment of a particular class (cell attachment proteins which contain the sequence Arg-Gly-Asp) in activity of both cell types. Development of this class of proteins, or related compounds, for modulation of skeletal metabolism will be discussed.

DEVELOPMENT AND STRUCTURE OF BONE

Bone is developed from undifferentiated mesenchyme (except in the case of the mandible which develops from neuroectoderm) via two pathways (1). During intramembranous formation, bone is formed directly from osteogenic precursors while in endochondral formation, bone replaces a cartilaginous model that serves as a template. During early stages of bone formation, cells that are responsible for the synthesis and deposition of bone matrix (the osteoblasts) perform their task in a somewhat unorganized fashion giving rise to woven bone. As growth and development proceeds, the initial bone is removed and remodeled by osteoclasts, a process termed bone resporption. Bone resportion is dependent on the presense of vasculature to bring osteoclastic precursors, which belong the the macrophage-monocyte series, into the area. It is now well established that the formation of mature osteoclasts is dependent on secretion of factors synthesized by osteoblasts, such that the two processes of formation and resorption are "coupled" (2, 3) After removal of bone, osteogenic precursors repopulate the area and either reform woven bone, or deposit bone in a more organized fashion resulting in lamellar bone, which incorporates into it a complex network of cells via Haversian channels.

Whether it be intramembranous or endochondral bone formation, the basic process of osteogenesis is similar. However, it need not be identical in that the cells that form bone are present in distinctly different microenvironments and may respond to different factors to carry out the same function. For example, in intramembranous formation, osteogenic cells are surrounded by undifferentiated mesenchyme, whereas in endochondral bone formation, calcified cartilage is also present. Furthermore, in the adult tissue, the process occurs within the environments created by the marrow cavity on the endosteal surface of bone. However, distinctions between these different sites of bone formation with respect to bone metabolism have not been well defined to date. The composition of the extracellular matrix that becomes calcified during osteogenesis may vary slightly from site to site, however, the presence of the primary structural elements appears to be universal.

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Bone is composed of water (20%), an inorganic phase (77%) representing mineral and other ions, and an organic phase (23%). The organic matrix (4,5), like other connective tissues, contains collagen, the primary structural protein of mammalian tissues. However, in bone, collagen contributes 90% of the total organic composition as opposed to between 10 and 30% in soft connective tissues. The collagen component is somewhat unique in that it is composed of virtually all type I collagen, with only trace amounts of other types. In addition, bone collagen bears different posttranslational modifications (carbohydrates and crosslinks).

The remaining 10% of the structural organic matrix is composed of a variety of other classes of macromolecules. Bone matrix contains several different types of proteoglycans. Proteoglycans contain a protein core (different gene products for different types of proteoglycans) to which one or more glycosaminoglycans are covalently attached. There are several types of glycosaminoglycans, which are long chain carbohydrates, that are determined by the type of repeating disaccharide unit (many of which are sulfated). A large proteoglycan bearing chondroitin sulfate glycosaminoglycan side chains, which may be related to a soft connective tissue proteoglycan called versican, is present in matrix that is destined to become bone. As bone development proceeds, it is replaced by two small proteoglycans, decorin and biglycan, which bear chondroitin sulfate glycosaminoglycans in bone, but dermatan sulfate in soft connective tissues. Decorin, so named for its ability to "decorate" collagen fibrils, is ubiquitous in soft connective tissues and in bone, while biglycan (containing two glycosaminoglycan side chains) is found at distinct pericellular locations during morphogenesis, and is highly enriched in osteoid (unmineralized bone matrix) and in lacunae where mature ostocytes reside. Although they have distinct histological localizations, analysis of their genes indicate that they are highly related and are composed of a repeating 24 amino acid leucine-rich sequence. Both have the ability to bind the multi-functional growth factor TGF-beta, pointing to a potential role in regulating this growth factor's activity. Hyaluronan, a glycosaminoglycan that is not attached to a protein core, is also present in bone during early stages of bone formation.

There are a number of glycoproteins that can be extracted from mineralized matrix, and these proteins are often sulfated and/or phosphorylated. Alkaline phosphatase, usually bound as an ecto enzyme to cell membranes, can be shed into the matrix where it is presumed to play a role in the matrix mineralization process. One of the most abundant bone matrix proteins, osteonectin, is somewhat unique in that it contains one and possibly two EF-hand high affinity calcium binding sites, as well as up to 10 low affinity sites. Including collagen, there are at least 5 and possibly up to 7 glycoproteins that contain the cell binding consensus sequence, Arg-Gly-Asp (RGD). These are thrombospondin, fibronectin, osteopontin, bone sialoprotein, and possibly vitronectin and BAG-75 (6). These proteins will be discussed in greater detail below.

Bone matrix contains another group of proteins that are posttranslationally modified to contain gamma-carboxy glutamic acid, indicative of sequences that are similar to those found in the vitamin K dependent blood coagulation factors that are also gamma-carboxylated. Matrix gla protein is found in many connective tissues other than bone, whereas osteocalcin is highly bone specific, and produced during the final stages of bone formation. Protein S, synthesized mainly by the liver, has also recently been identified in bone matrix (7). The remaining organic fraction is composed of low abundancy proteins that most likely do not play a structural role, including growth factors, enzymes and their inhibitors, proteolipids, etc.

The mineral component of bone is composed of an impure hydroxyapatite that contains carbonate in addition to calcium and phosphate, which causes the crystals to be somewhat smaller than what is formed by calcium and phosphate in vitro. Hydroxyapatite deposition may occur through unstable intermediates such as amorphous calcium phosphate or octacalcium phosphate, and is mediated either by membrane bound structures (matrix vesicles) or by deposition via non-collagenous components onto the collagenous scaffolding.

Although bone tissue contains many different cell types, including hemopoietic and stromal cells of the marrow and cells associated with the vasculature, mineralized matrix is mainly the product of cells in the osteoblastic lineage. During bone formation, cells in this lineage pass through a series of maturational steps that can be identified both in vitro and in vivo (5,8,9). The biosynthetic products of the cells are somewhat stage specific with early cells (preosteoblasts)

synthesizing collagen, decorin, thrombospondin, fibronectin, and alkaline phosphatase. More mature osteoblasts also synthesize osteonectin, biglycan, osteopontin, and bone sialoprotein. Osteocytes maintain synthesis of biglycan and begin to synthesize osteocalcin. In addition to the proteins synthesized endogenously, a long list of proteins of exogenous sources are adsorbed to hydroxyapatite and become entombed in mineralized matrix. For example, serum albumin and alpha$_2$-HS glycoprotein are both synthesized in the liver, but are concentrated in bone up to 100 times the level present in the circulation. There are also a number of growth factors that are synthesized endogenously and exogenously, but it is not clear if there are differences in the activity of a particular factor depending on the source (5).

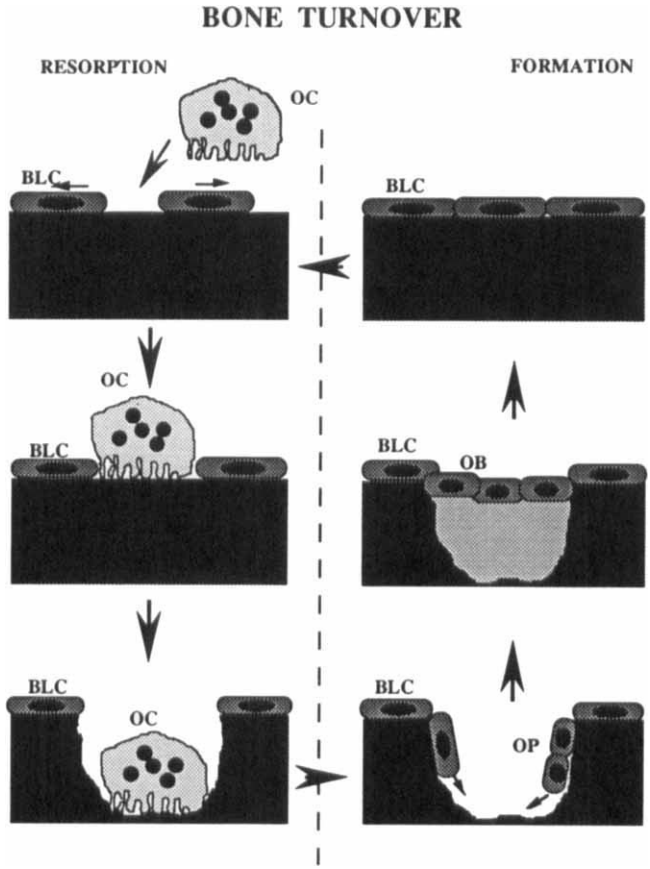


Figure 1. Model of Bone Turnover. During adult life bone is constantly undergoing bone turnover, which is the coupling of two opposing forces; bone resorption and bone formation. Remodeling begins when bone lining cells (BLC) on the surface of mineralized matrix retract such that an osteoclast (OC) can attach to the matrix via integrins. This cell matrix interaction induces the formation of an extracellular lysosomal compartment, the means by which bone matrix is degraded. Once the osteoclast has resorbed a certain amount of bone and has moved away, osteogenic precursors (OP) repopulate the area and mature to form osteoblasts (OB) which begin to synthesize and deposit mineralized matrix. Once the gap has become filled, the cells become inactive until another round of turnover is initiated. Figure is courtesy of Dr. W.J. Grzesik, NIDR, NIH.

The obvious difference between bone and soft connective tissues is the presence of hydroxyapatite, and it was the hope of pioneer researchers in the field that deposition of this mineral would be due to the presence of a unique set of proteins. However, it is apparent that the mineralization process in vivo is not so straightforward. It is now recognized that the pattern of proteins isolated from mineralized bone matrix and synthesized by cell culture model systems that faithfully maintain the osteoblastic phenotype is remarkably similar to the pattern of proteins synthesized by fibroblasts of soft connective tissues (5,8,9). To date, only two mineralized matrix proteins have been identified that are bone-specific; bone sialoprotein (BSP), which is found in certain hypertrophic chondrocytes of calcified cartilage, osteoclasts (10) and osteoblasts, and osteocalcin (11,12) found in osteocytes. However, closer examination of the chemical nature of the mineralized matrix indicates two factors of potential relevance: 1) the ratio of the components in mineralized matrix is different from that of soft connective tissues; and 2) mineralized matrix proteins are structurally different, due to either differential splicing of RNA precursors following gene transcription, differential post translational modification, or proteolytic processing during secretion and deposition. It is these differences that make it feasible to develop synthetic analogs with tissue specificity for potential medicinal intervention.

THE RGD-CONTAINING PROTEINS OF BONE

This group of proteins, or analogs, which support cell attachment activity represents a class of molecules that could be potentially manipulated to influence bone metabolism. After initial formation, bone is constantly going through cycles, termed bone turnover (Figure 1), of bone resorption by osteoclasts, followed by bone formation by cells in the osteoblastic lineage. These two phases must remain "coupled" in order to maintain homeostasis. But in certain disease processes, resorption and formation become uncoupled leading to a loss of bone (e.g., osteoporosis) or too much bone (e.g., Paget's disease of bone). From in vitro and in vivo studies, it is recognized that both precursors and mature forms of osteoclasts and osteoblasts are dependent on interactions with matrix components for normal activity. Although the RGD-containing proteins are produced at somewhat different stages of maturation, it is not clear why there are so many of them in mineralized matrix and what role they play in bone turnover.

Collagen - This large fibrous protein is composed of three polypeptide (alpha) chains of M_r 95,000 that are coiled together to form a stable triple helical molecule. There are several RGD sequences within the collagen molecule, as well as non-RGD sites that mediate cell attachment (13,14). It is clear that under in vitro conditions (binding of the protein to bacteriological plastic), the RGD is active in mediating cell attachment, although other sequences may also be operative. However, this may not be the case in vivo. It is not known what conformation the RGD regions take on when wound into the triple helix. In addition, type I collagen fibrils are often coated with other molecules including types III, V, and FACIT collagens, and decorin. Consequently, the RGD may not be available to interact with cells. By in vitro assays, osteoclasts do not attach well to collagen (15), and while osteoblastic cells do attach, it is in a predominantly RGD-independent fashion (16).

Thrombospondin - This trimeric molecule with disulfide-linked subunits of ~M_r180,000 (17), binds to a number of extracellular matrix components including collagen, laminin, and heparin. It is produced during early phases of bone formation. The RGD sequence is present in the globular domain at the C-termini of the subunits. The conformation of this region is dependent on Ca^{2+} and it is probable that it may not be available for cell attachment activity under most conditions. Other, non-RGD cell binding regions have been identified in the central stalk portion of the subunit and in the N-terminal globular domain. In vitro, thrombospondin has been found to inhibit osteoclast attachment (15), and mediates cell attachment, but not spreading in normal osteoblastic cells (16). Interestingly, this attachment is not inhibited by an RGD peptide, indicating that another sequence in the molecule is responsible.

Fibronectin - The fibronectin molecule consists of two similar, but non-identical ~M_r 200,000 subunits that are disulfide bonded near the C-termini, and contain numerous functional domains that are made of repeating sequences of three types I, II, and III (18). In one particular type III repeat, there is a change in sequence resulting in Arg-Gly-Asp. Structural analysis by NMR spectroscopy has predicted that the RGD is solvent-exposed on a conformationally mobile loop connected to a compact hydrophobic core (19). Present in milligram amounts in serum, fibronectin is also

produced during early stages of bone formation and mediates osteoclast and osteoblastic cell attachment in vitro (15, 16). Unlike other connective tissue fibroblasts which are inhibited by RGD peptides, the attachment of bone-forming cells to fibronectin is RGD-independent and must rely on other sequences in the molecule (16).

Vitronectin - Composed of a single polypeptide of M_r ~75,000, vitronectin is the major serum protein that binds to tissue culture plastic under usual conditions, a fact that may lead to selective growth of cells that utilize this attachment protein (16). In depth structural analysis of the RGD region in this protein has not been done, but by using simple computer analysis (GOR plots), it is predicted that in this protein, the RGD is not on a loop, but rather at a 90^0 bend in the molecule (5). It is probable that this is a feature that distinguishes binding of fibronectin and vitronectin to different receptors. Vitronectin is found in mineralized matrix (15), and may be synthesized by osteoblastic cells. It is active in mediating attachment of osteoclasts and osteoblasts in vitro. However, cell attachment to vitronectin can be distinguished from that to fibronectin in that it is blocked to a greater extent by cyclic RGD peptides than by linear peptides (20).

Osteopontin (Spp, pp66, 2ar, BSP-I) - This RGD-containing component has a protein backbone of M_r ~44,000, and is highly post translationally modified by the addition of N- and O-linked oligosaccharides containing sialic acid, and is often phosphorylated (21). Computer analysis predicts a conformation of the RGD region (which is in the middle of the molecule) similar to that of fibronectin (5, 22, 23). While it is synthesized in osteoblastic cells, it is found in a variety of other cell types such as renal epithelial cells, mammary epithelial cells, and certain monocytic cells in bone marrow, and has been found to be induced by transformation events. Osteoclasts also appear to synthesize osteopontin (24), and both osteoclasts (15) and osteoblastic cells utilize it for attachment (16, 25).

Bone Sialoprotein (BSP) - Along with osteocalcin, BSP is one of the two mineralized matrix specific proteins although it has been identified in cytotrophoblasts of placental membranes, which are cells that fuse to form a syncytium, and often mineralize at late stages of gestation (10). The protein, M_r ~55,000, is approximately 50% carbohydrate (12% sialic acid). Sequence analysis suggests that the RGD region, which is near the C terminus of the molecule, resembles that of vitronectin (5, 23) and revealed numerous tyrosine sulfation consensus sequences flanking the RGD. In fact, BSP has been identified as a sulfated protein in several animal species (26, 27), but it is not known what influence sulfation plays on its activity. This protein colocalizes with the first deposits of hydroxyapatite detectable at the EM level in structures that appear to be preorganized by osteoblasts (28). Consequently, it may play multiple roles in mediating matrix mineralization, and cell attachment of both osteoclasts (15) and osteoblasts (16, 25). In addition to the RGD sequence, a fragment that does not contain RGD, generated by either endogenous protease or with chymotrypsin, also mediates osteoblastic attachment (29), but it is not known whether it supports osteoclast attachment.

It is apparent that when cells utilize them as a substrate, the RGD-containing bone matrix proteins are active in roughly the same molar concentrations, which would imply one binding site per molecule. In osteoclasts, there have been few studies that distinguish between RGD dependent and independent attachment, but in osteoblastic cells it is clear that the RGD sequence is not the only active site in collagen, thrombospondin, and fibronectin (16). This information suggests that there are different cell surface receptors on bone cells that convey different matrix binding properties.

INTEGRINS

This family of receptors is composed of alpha and beta subunits that are disulfide bonded to one another (20, 30). The subunits possess short cytoplasmic tails, transmembrane regions and lengthy extracellular stretchs, that together form the binding site for the matrix ligand. There are up to fourteen different alpha subunits, that can combine with up to eight different beta subunits. The combination of different alpha subunits to beta subunits, in some cases, conveys ligand specificity but in general there are a range of ligands that can be bound by a particular integrin (20, 30). The integrins that are present in bone are beginning to be identified with monospecific reagents such as antibodies and cDNA probes.

Osteoclasts - Bone resorption is accomplished by a highly specialized cell, the osteoclast, that is formed by blood-borne precursors from the monocyte-macrophage series. Osteoclasts must attach to bone matrix destined to be degraded, establish polarized secretion of acid ions and lysosomal enzymes, and finally move away from the newly resorbed area to allow osteoblastic precursors to repopulate the excavated portion (3). All of these steps are dependent on cell-matrix interactions. In vitro studies suggest that mature osteoclasts attach to osteopontin and bone sialoprotein (15, 31, 32, 33), which results in the organization of the osteoclastic membrane into three regions: the ruffled border which is highly convoluted cell membrane that secretes high concentrations of acid ions and lysosomal enzymes, and which is sealed from the external environment; the "clear zone" or the sealing zone (34, 35); and the basolateral membrane that faces the marrow cavity. In the sealing zone, microfilamentous structures have been postulated to mediate tight attachment (36, 37), and in vitro, these microfilamentous structures have been termed podosomes (38) and have been found to contain vinculin, talin, and gelsolin, as well as actin (39, 40).

The presence of integrins on osteoclasts was first brought to light through the use of two monoclonal antibodies raised against isolated osteoclasts, that were found to identify the $alpha_V beta_3$ integrin (41), which was also identified in a number of other studies (31, 32, 42-54). This integrin binds to vitronectin, fibronectin, thrombospondin, collagen, osteopontin, and bone sialoprotein, and it was postulated that it mediated tight attachment to osteopontin and/or BSP (31,35). However, other studies (46) have failed to identify this receptor in the "clear zone"

The $alpha_2$ and $beta_1$ subunits which combine to form an integrin that binds to collagen and laminin have also been identified on osteoclasts(15, 44, 55-57). It is possible that other integrins are present since the $beta_1$ subunit can combine with a number of different alpha subunits ($alpha_{1\text{-}7,V}$) and that each combination has slightly different ligand binding properties.

Although it is not entirely clear which integrins are involved during the different stages of osteoclast formation or activation, the evidence suggests that cell-matrix interactions are involved in establishing osteoclastic activity. The intracellular pathways by which the consequence of matrix-integrin interactions alter cellular metabolism are beginning to be investigated. It has been found that binding of osteoclasts to either osteopontin or bone sialoprotein, or peptides derived from the RGD regions causes a reduction in intracellular Ca^{2+} levels in chicken osteoclasts (58). It has previously been determined that increases in intracellular calcium levels cause a decrease in podosome formation (58). It is tempting to speculate that one of the consequences of $alpha_V beta_3$ binding (the lowering of intracellular Ca^{2+}) results in an increase in podosome formation. There is also evidence that receptor binding activates tyrosine kinase, which can also affect podosome formation (59, 60).

Osteoblastic Cells - Bone formation by cells in the osteoblastic lineage occurs in a number of related, but non-identical patterns that differ from one another by the time and place in which they are occurring (5). During embryonic development intramembranous bone formation occurs within loose interstitial mesenchyme, which is gradually replaced by bone, whereas during endochondral bone formation osteogenesis occurs on top of a cartilaginous model. In both cases, vascular invasion is a process that is intimately associated with osteogenesis, and brings in osteoclastic precursors that penetrate through the vessel wall to initiate bone turnover. Although the basic pattern of bone formation is the same, it is occurring in different microenvironments (neighboring cell types, physical forces, etc.), and the factors that influence osteoblastic metabolism may not be identical in the different microenvironments. However, during turnover of the adult skeleton, it is quite clear that cell-matrix interaction is required for bone formation to proceed following bone resorption. Once an osteoclast has moved away from a resorption lacuna, osteogenic precursors, most likely recruited from the marrow stromal population or an activated lining cell, must migrate into the area and begin to proliferate and differentiate through the maturation pathway.

Due to the limited number of reagents with high specificity, it has been difficult to determine the entire integrin content of cells in the osteoblastic lineage with any certainty. By immunohistochemistry in developing long bone, the $alpha_4$ subunit (which can combine with $beta_1$) and $alpha_5 beta_1$ have been identified (16), both of which can bind to fibronectin. An

antibody against alpha$_v$, and one that recognizes beta$_3$ and/or beta$_1$ also stained bone-forming cells. However, quantitative differences between cells at different stages of maturation are difficult to determine by immunohistochemistry, which is only semi-quantitative at best. Quantitative types of analysis will require more precise methodologies to determine when during bone formation, and at what stage of maturation the various integrins are expressed and functional.

There have been even fewer studies on changes in osteoblastic metabolism dependent on attachment to different substrata. It was found that attachment to thrombospondin and collagen slightly stimulated cell proliferation, whereas no proliferation was noted on osteopontin or bone sialoprotein (16). However, there was an increase in alkaline phosphatase, a hallmark of the osteoblastic phenotype, with increasing number of days in culture on osteopontin and bone sialoprotein. Interestingly, this increase in alkaline phosphatase as detected by a histochemical stain was coincident with decreased viability of the cells which may indicate a correlation between histochemical alkaline phosphatase and apoptosis (programmed cell death). No studies identifying intracellular signalling pathways in non-transformed osteoblastic cells have been reported, but occupancy of beta$_1$ was correlated with an increase in alkaline phosphatase activity in an osteosarcoma cell line (61).

POTENTIAL THERAPEUTIC USE OF RGD-CONTAINING PROTEINS OR ANALOGS

As mentioned above, there are two phases of the bone turnover cycle, bone resorption and bone formation, that must remain coupled to maintain normal bone density. Any process that results in an imbalance between these two opposing forces results in disease and skeletal dysfunction.

Bone Resorption - Although on the surface, it would appear that bone resorption is an unnecessary evil, it is required to liberate calcium and phosphate as needed by the body, and to rejuvenate the skeleton by removing areas fatigued by microfracture. There are a number of ways that both osteoclastic and osteoblastic abnormalities result in either decreases or increases in bone resorption. In cases where it is decreased, osteopetrotic-like syndromes arise, where marrow cavities are gradually filled in and hemopoiesis is obliterated. When bone resorption is increased without a concomitant increase in formation, there is a decrease in bone mass, as exemplified by osteoporosis, humoral hypercalcemia of malignancy, disuse osteoporosis, and a number of other conditions. It is this type of disease process that may be amenable to treatment with RGD-containing proteins or peptides, since it has been shown that osteoclasts utilize at least some of these proteins for cell attachment.

There are a number of systems in which RGD-containing proteins or peptides can be tested for their ability to affect bone resorption: by purifying osteoclasts and measuring pit formation after incubation (62); purifying osteoclasts and plating them onto either protein coated surfaces (15, 49, 50) or prelabeled bone tissue (32, 63); or by using bone rudiments at different stages of development (either when osteoclastic precursors or mature osteoclasts are present (64).

In all of the systems, it was found that a number of RGD-containing peptides had effects on either cell attachment or bone resorption. The smallest peptide, RGD, did not have an effect on bone resorption (46), but the peptide RGDS inhibited cell attachment (59) at IC_{50} 26 μM and resorption at IC_{50} 0.1 mM (32). The linear peptide, GRGDS, inhibited cell attachment (54) but did not affect resorption (64) or inhibited at relatively high concentrations (32). However, peptides that had been modified or cyclized to take on a particular conformation were far more active in inhibiting cell attachment at IC_{50} 7.0 μM (54) or bone resorption (64). It has been previously determined that linear peptides bind preferentially to the fibronectin receptor while the cyclized derivatives bind to the vitronectin receptor (20). The results suggest that the vitronectin receptor is actively involved in osteoclast attachment as well as actual bone resorption. This is further confirmation of studies in which antibodies against the vitronectin receptor were found to inhibit bone resorption (44, 47, 57). Furthermore, in developmental studies, it was found that peptides influenced preosteoclast development but were not capable of inhibiting resorption of mature osteoclasts (64).

A family of proteins termed "disintegrins" has been isolated from the venom of different types of vipers and include echistatin (49 amino acids), kistrin (68 amino acids), applaggin

(17.7 kDa), and trigramin (7.5 kDa). These proteins are highly homologous and contain a high content of cysteine and the RGD sequence. Interestingly, the overall secondary structure of the proteins is similar with a compact hydrophobic core and the RGD extending in a somewhat mobile loop (similar to the RGD loop in fibronectin), however, the conservation of disulfide bridge formation is very low in spite of the high conservation of the cysteine residues within the sequence (65-68).

Interest was first drawn to this group of proteins due to their ability to inhibit platelet aggregation by binding the GP IIb/IIIa, which is a $beta_3$ type of integrin. Based on these findings, echistatin in particular has been investigated and found to inhibit osteoclast attachment to protein coated substrates (54) at IC_{50} 0.78 nM, and bone resorption (32, 64) at IC_{50} 0.1 to 1 nM. Kistrin was also active (64). Echistatin was found to colocalize with vinculin and "an alpha-like subunit" on the osteoclast membrane (32). These studies suggest that disintegrins or their derivatives are active at low concentrations and may be useful in inhibition of bone resorption.

Bone Formation - Since it can be observed that the administration of RGD-containing proteins and peptides in solution has effects on bone resorption, it is unlikely that they would be beneficial in stimulating formation, and in fact they have been noted to have a deleterious effect (69). However, it is apparent that when they are immobilized they provide a suitable environment for cell attachment and in some cases proliferation (16). For this reason, it should be possible to use these proteins or peptides in combination with biomaterials that would have broad usage in reconstructive surgery.

Currently, there are a number of biomaterials being utilized in dental and orthopaedic surgeries including titanium, hydroxyapatite, and ceramics and polymers of varying composition. The major downfall of these implants is that in the majority of cases there is failure of bonding at the tissue-implant interface. In early studies, this failure was due to the fibrous nature of the tissue surrounding the implant, and current studies are aimed at obtaining biointegration such that bone is formed directly on the surface. While there have been advances in this area, success is not uniform. It could easily be envisioned that covalent linkage of the appropriate cell attachment protein(s) or derivative may encourage the ingrowth, proliferation, and subsequent maturation of appropriate stem cells to obtain more rapid and efficient integration of the implant. However, it is clear that much more basic information is needed. For example, there are considerable differences between the sequences surrounding the RGD sequence in many of these proteins and the effects of tyrosine sulfation and serine and threonine phosphorylation on binding properties have not been investigated. It is also not known if there are major changes in the integrin profile as the cells pass through the maturational stages. Consequently, development of modified implant materials represents a new frontier in the application of basic information to clinical practice.

Summary - In conclusion, the last decade has witnessed an explosion in our knowledge of bone matrix proteins, and in particular, the potential function of RGD-containing proteins in both bone resorption and bone formation. This basic knowledge is now being applied in the area of bone resorption through the use of RGD-containing peptides and proteins purified from snake venoms, and there is a potential for using the matrix proteins or peptides as a means of bonding to implant materials. However, there are potential pitfalls to the widespread use of such preparations. The receptors to which they bind are present on a wide variety of cell types including endothelial cells and platelets. In addition, there have been no *in vivo* studies to confirm ligand-integrin specificities that have been determined by organ culture or cell culture experiments. Finally, it is apparent that there are many variations of a particular type of receptor; e.g., there have been at least five variants of the vitronectin receptor reported to date (54). Further studies are required to specifically determine the integrin profile of each particular cell of interest. However, it is this variation, along with the variations in the RGD-containing bone matrix proteins that may ultimately allow us to generate reagents unique for a certain cell type, in a particular matrix environment at a specific stage of differentiation, in order to manipulate the processes of bone resorption and formation.

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Chapter 25. New Approaches to Non-Immunogenic Monoclonal Antibody Cancer Therapies

Lois M. Hinman[1] and Geoff Yarranton[2]
[1]Medical Research Division, American Cyanamid Company, Pearl River, NY. 10965
and [2]Celltech, Ltd. Slough, UK

Introduction - Since the development of hybridoma technology to produce monoclonal antibodies (MoAbs) in 1975, much research has been directed at the design of MoAb-based therapies for human disease, particularly for the treatment of cancer. Now, eighteen years later, despite a great deal of research, only a small number of MoAb-based products have been approved for clinical use including OKT3, a murine IgG for treatment of renal transplant rejection, and Myoscint, an ^{111}In labeled murine antimyosin MoAb for imaging of myocardial damage (1), but no products for cancer therapy. In 1992, the first MoAb-based diagnostic agent for tumor imaging, OncoScint CR/OV, a murine MoAb, B72.3, linked to ^{111}In for γ-camera imaging of colorectal and ovarian cancers received FDA approval (2) and currently a number of antitumor MoAb-based therapeutics are in clinical trials (3,4).

Frustrations which have been encountered in the development of MoAb-based therapies for cancer are numerous due to a number of fundamental technical problems including: insufficient potency of the delivered agent, low specificity for tumor vs. normal tissue, poor penetration of the macromolecular conjugates into solid tumors, antigenic heterogeneity in tumors, and the inherent immunogenicity of the murine MoAbs and MoAb-based products (5, 6). The significance of most of these problems varies among therapeutic approaches and tumor targets, however, immunogenicity is a general concern that limits the therapeutic possiblities for murine MoAbs or MoAb-based products in any protocol requiring multiple or repeat injections. Immune responses in the form of HAMA (human anti-mouse antibodies) often result from even a single injection of murine-based MoAbs in all but the most immunosuppressed patients. As repeated doses are expected to be required for most treatment regimens in anticancer therapy, a great deal of effort has recently been directed toward the engineering of less immunogenic antibody constructs. Current approaches to preparing non-immunogenic MoAbs include the engineering of rodent antibodies ("humanization"), and the development of high affinity human MoAbs. The first humanized MoAbs are now either in or about to enter Phase I clinical studies for the treatment of a variety of cancers including: (i) a potent drug conjugate of hCTM01 for ovarian cancer (7), (ii) a radioisotope conjugate of M195 for the treatment of acute myelogenous leukemia (AML) (8), and (iii) an unconjugated humanized version of Ab4D5 for drug sensitization in the treatment of breast cancer (9). These trials will undoubtedly provide further information on the properties of humanized antibodies and their likely efficacy in cancer therapy. The strategies being used to prepare humanized and high affinity human MoAbs for therapy are outlined below.

Whether or not humanized antibodies will operate through their effector functions or require radioisotopes or cytotoxic agents conjugated to the protein remains to be resolved (10). In order to exploit the tumor targeting capability of MoAbs for effective cancer therapy, investigators have conjugated cytotoxic agents, radionuclides, clinically approved chemotherapeutics, and highly potent protein toxins to the MoAbs for delivery to tumors. The combined experience from numerous studies with targeted therapy suggests that (i) the level of antigenic expression for the carrier MoAb on a target cell, (ii) the mode of release of the toxic agent from the MoAb, (iii) the specificity of the MoAb for the target cell, and (iv) the degree and rate at which the MoAb is internalized into the target cell all must be considered in the design of an effective targeting system. Each therapeutic approach has unique problems. For radioisotope conjugates the specificity of the MoAb, its half life in the serum and its biodistribution in humans are key concerns while for drug-MoAb conjugates, low potency of the drug and lack of internalization of the carrier MoAb can be serious problems. One promising MoAb-based therapeutic approach is immunotoxin (IT) therapy. IT's are prepared by linking MoAbs or MoAb fragments with phytotoxins, potent ribosomal inhibiting proteins of plant or bacterial origin that are on the order of 1000-fold more potent than standard

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chemotherapeutics and act catalytically rather than stochiometrically on their cellular targets (11). Several IT's have shown promising preclinical results and have recently been the subject of clinical trials in breast, colon, ovarian carcinomas, melanoma, and a number of hematopoetic malignancies (12, 13, 14, 15). However, the major problem with IT therapy involves the significant immune responses to both the protein toxin and MoAb components of conjugates which is dose-limiting in all but the most severely immunocompromised patients(16).

The limited success with IT's shows that targeting agents too toxic to be developed as stand alone therapies have great potential, particularly if less immunogenic therapeutic modalities than the phytotoxins can be identified. Accordingly, one new strategy for MoAb-based therapies currently receiving attention is the conjugation of highly potent low molecular weight drugs which, like the phytotoxins, are up to 1000-fold more potent than traditional chemotherapeutic agents and too toxic as stand-alone therapies, yet, unlike the phytotoxins, are small non-proteineous molecules (M Wt <2,000). The conjugation of several such highly potent cytotoxics to MoAbs has recently been reported and the chemistry and status of preclinical evaluations of these potent MoAb-drug conjugates are described below.

NON-IMMUNOGENIC MONOCLONAL ANTIBODIES

Higher mammals have five classes of immunoglobulin, termed IgG, IgM, IgA, IgE, and IgD. The structure and function of these are conserved across species (17). Nearly all those used for cancer therapy have been of the IgG class. Clinical experience with mouse antibodies is that the majority of patients mount a human anti-mouse antibody (HAMA) response after a single injection which limits the utility of further doses (18). In some patients, up to 60% HAMA represents anti-idiotype (against the variable region) (19) and may lead to Ab3 antibodies (anti-anti-idiotype) with anti-tumor activity (20).

Engineering Rodent Monoclonals - The inability to give multiple dose therapy due to HAMA is a significant problem for antibody targeting of radioisotopes or cytotoxic drugs to tumors. Chimeric (rodent variable - human constant region) antibodies have been constructed, in order to overcome this problem. Some chimeric antibodies that have been evaluated in the clinic as cancer targeting agents are listed in Table 1. It is clear from these clinical studies that cancer patients do raise an immune response to chimeric antibodies. The response varies from antibody to antibody, suggesting that some rodent variable domains are more immunogenic than others. For example, in 23 metastatic colorectal adenocarcinoma patients treated in three studies using c17-1A(γ_1) antibody, only one patient generated an immune response (21). In contrast, murine 17-1A induced HAMA responses to the variable region in all patients studied(20). Another chimeric MoAb, cB72.3(γ_4) has been shown to induce anti-antibody responses in 50-75% of colorectal cancer patients (7) tested and in strong responders the antibody response was confirmed to be to the cB72.3 variable region (22). Interestingly, these results suggest that antibody variable regions differ in their immunogenicity since the variable region was immunogenic in cB72.3, yet less so for c17-1A. With c17-1A, it would appear that the human constant region reduced the immunogenicity of a mouse variable region.

In an attempt to overcome the remaining immunogenicity of rodent antibody variable regions, extensive manipulations have been carried out such that the resulting antibody more closely resembles human antibodies. This process was originally known as complementarity-determining region (CDR) grafting (23), in which the hypervariable loops (or CDRs) from the heavy and light chains of the rodent antibody that are responsible for antigen binding were used to replace those of a recipient human antibody. The extent of the CDR loops was determined by amino-acid sequence comparison between antibodies and by structure modeling. Further developments of this methodology aimed at recovering more of the parent antigen binding activity have been made, including reshaping (24,25), hyperchimerization (26,27,28), civilization (29), and veneering (30). These latter methods take into account the need to maintain CDR structure within the human antibody background. To achieve this, key framework residues, non-contiguous with the CDRs in linear sequence, are transferred from the rodent antibody. For example, the anti-lymphoma monoclonal antibody CAMPATH-1 initial CDR-graft recovered only 2.56% of the murine antibody affinity. Subsequent framework residue changes improved its activity (25). Similar results have been obtained with anti-cancer monoclonals B72.3 (31) and A425(32). The method of humanization is now well established and activities in excess of 50% of the starting murine antibody are routinely achieved. A large number of humanized anti-cancer MoAbs are currently in development or early phase clinical trials (Table 1).

Published data on the immunogenicity or efficacy of humanized antibodies is limited. Three studies with the humanized CAMPATH-1 (CAMPATH-1H) antibody have been published (33,34,35), one of these in cancer therapy. Two patients with non-Hodgkin lymphoma were treated with CAMPATH-1H for up to 43 days with escalating doses ranging from 1-20 mg/day by intravenous infusion (31). No anti-antibody responses were detected during the treatment. In both patients lymphoma cells were cleared from the blood and bone marrow and splenomegaly resolved. The same antibody has been used as a therapy for rheumatoid arthritis (33) in 8 patients and clinical benefit was observed in 7. Anti-antibody responses were detected on repeat treatment, although these were low titer anti-idiotypic responses.

Primatized MoAbs - Immunoglobulin variable region genes cloned from non-human primates (cynomologus macaques), have been shown to have 85-98% homology with human sequences. Antibody genes have been isolated from monkeys immunized with human CD4 and a monkey/human chimeric antibody with high affinity (3.2×10^{-11} M) has been constructed(36). These data suggest that this is a viable approach for preparing MoAbs of high affinity to human antigens, and could be used to raise antibodies to human tumor antigens.

TABLE 1

ENGINEERED ANTIBODIES FOR CANCER THERAPY

ANTIBODY	ANTIGEN	IMMUNE RESPONSE	TUMOR TYPE	REFERENCE
		CHIMERICS		
17-1-A	TAA	Anti-id (<4%)	Colorectal	21
B72.3	Tag-72	Anti-id (40-60%)	Colorectal	22
L6	TAA	Anti-id (32%)	Lung / Breast	37
14.18	GD2	Anti-id (50%)	Melanoma	38
		HUMANIZED		
H17 E2	PLAP		Ovarian	39
425	EGFR		Astracytoma / others	31
4D5	$p185^{HER2}$		Breast	9
A5B7	CEA		Colorectal	7
BW431/26	CEA		Colorectal	7
B72.3	Tag-72		Colorectal, Ovarian	7
CTM01	PEM		Breast, Ovarian	7
A33	TAA		Colorectal	7
Anti-Tac	CD25		ATL	26
M195	CD33		AML	28
P67.6	CD33		AML	7
YTH 35.5HL	CDW52	Anti -id	Lymphoma	24
		HUMAN		
L72	GD2	Anti-id?	Melanoma	42
16.88	TAA		Colon	40

Human Monoclonals - Human monoclonal antibodies may be preferred to humanized rodent antibodies if the latter fail to solve the problem of immunogenicity. Clinical experience with human anti-tumor MoAbs is limited due to the problems associated with raising high-affinity human antibodies. One study carried out in colorectal cancer patients, with a human IgM monoclonal antibody 16.88, demonstrated good tumor localization and no anti-antibody response (40). This antibody was developed from peripheral blood lymphocytes of a colorectal cancer patient (41). A second human antibody, L72, reactive with ganglioside GD2 has been evaluated in melanoma patients. Regression of cutaneous melanoma following intralesional injection was demonstrated.

Human anti-human antibodies were detected in 2 out of 5 patients which blocked antigen binding (42). Recently several new technologies have been developed that may enhance the prospects for obtaining human monoclonal antibodies, these are: (i) recombinatorial phage libraries (Figure 1) and (ii) in vitro immunization.

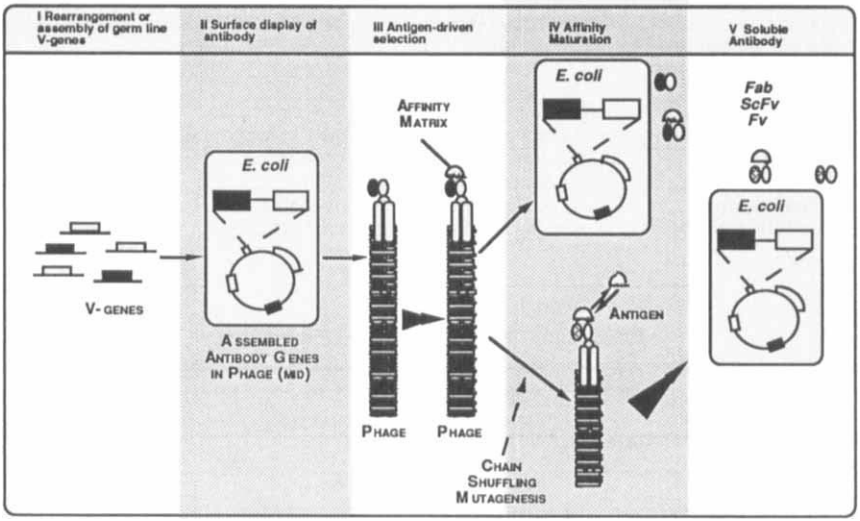


Figure 1: Schematic of the bacteriophage display methodology for the identification and selection of high affinity monoclonal antibodies from combinatorial libraries. The V-gene segments (I) from the genome are cloned into a bacteriophage expression vector allowing their expression on the surface of the phage (II). Those phage expressing V-gene pairs that bind antigen are identified by binding and elution from an affinity matrix (III). Increased affinity (affinity maturation) is achieved by exchanging V-gene partners between antibodies that bind to the same antigen (chain shuffling) or by mutagenesis (IV). By changing *E. coli* host strain, high affinity antibody is secreted into the medium and can be then purified (V).

Recombinatorial Libraries - It is estimated that 10-30% of B lymphocytes in normal, healthy individuals are engaged in making autoantibodies (43) . However, these autoantibodies tend to be of low affinity, and hence of little therapeutic value. In disease states, e.g. cancer, human monoclonals to self or tumor antigens can sometimes be isolated (37, 44). These antibodies are often specific, but recognize a restricted range of epitomes (45). Recently several groups have generated libraries of human V-genes and expressed these in bacteria using filamentous phage technology (46,47,48,49). Expression of V_H and V_L genes as a single chain FV (scFV) or Fab molecules on the surface of phage, allows for selection of antigen binding properties through the use of purified antigen as an affinity purification matrix. By a process called "chain shuffling"(50), new combinations of V_H and V_L domains can be generated and selected in vitro. This process can be carried out with either mutated or non-mutated V genes and selection for higher affinity MoAbs can be applied by increasing the stringency of elution conditions from the affinity matrix (Figure 1). The use of this technology for the generation of anti-tumor MoAbs has recently been published (51). Low affinity antibodies to human (CEA) and the MUC-1 gene product were isolated from repertoires of rearranged V-genes derived from the mRNA of peripheral blood lymphocytes (PBLs) from non-immunized humans. Further improvements in affinity should be possible by a combination of mutation and further chain shuffling. This example therefore demonstrates the feasibility of the approach as applied to cancer antigens.

Using bacteriophage technology to generate human variable region domains that can subsequently be recloned into a human constant region gene fragment should allow the production of a variety of human anti-tumor antibodies. The questions that need to be answered in the future are: (i) can this approach be applied successfully to all antigens?; (ii) can high affinity

human MoAbs ($K_D=10^{-10}$ - 10^{-11}/ mol) be generated using complex antigens?; and (iii) if the V region genes are mutated and selected *in vitro*, will they be immunogenic in vivo? It is likely that these questions will be answered in the next 2-3 years. An additional application of this technology, that is particularly relevant to cancer therapy, is exemplified by a recent study with the anti-breast antibody L6 (52,53). Using phage display technology in combination with codon-based mutagenesis, an attempt was made to fine-tune the specificity of the L6 antibody. The nature of the L6 antigen is not fully characterized, although a gene encoding an antigen reactive with L6 has been isolated (54). To evaluate changes in the antibody specificity, the reactivity of the mutated V regions to bind a panel of three anti-idiotypic antibodies was evaluated. Elimination of the binding of one anti-idiotypic antibody was achieved, whilst maintaining reactivity with the other two. This result suggests that this technique may be useful for the removal of unwanted normal tissue reactivities in anti-tumour MoAbs.

In Vitro Immunization - Another route for the generation of human monoclonal antibodies is by primary, in vitro immunization of human splenocytes (55). Reports of such in vitro methodology for a limited number of antigens (e.g. ferritin), with affinities of ~ 10^7 - 10^8/mol, suggest that this may be a feasible route for human antibody generation. Questions remain concerning whether or not these responses are primary responses. The ability to combine this approach with the phage display technology may well circumvent the difficulties of immortalization of the immunized human B lymphocytes. A similar approach to the above, but using severe combined immunodeficiency mice that have been injected with human peripheral blood lymphocytes (SCID-hu)(56), may also yield human antibodies of high affinity. In this case, the reconstituted mice are challenged with antigen prior to the rescue of B cells for cell fusion or combinatorial phage library generation (57).

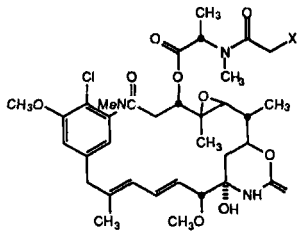
The technologies now available for the generation of human antibodies are most promising. A systematic study is needed to demonstrate definitively that these approaches can yield high affinity human monoclonal antibodies to a range of tumor antigens. Once the demonstration that primary responses and affinity maturation can be achieved, then the immunogenicity of such antibodies compared with those made by humanization technology should be determined.

PROGRESS TOWARD NON-IMMUNOGENIC TARGETED CHEMOTHERAPY

Considerations - Some of the earliest work in the field of MoAb targeting involved attempts to prepare conjugates with standard chemotherapeutic agents such as methotrexate, adriamycin, and vinca alkaloids for selective delivery to tumors (6, 58,59). In addition to HAMA, drug conjugates suffer from a number of specific problems, the most important of which is the lack of potency of standard chemotherapeutics which is further decreased by the chemical modifications required for MoAb attachment (60,61,62). One conjugate of doxorubicin linked by a thioether linkage to a chimeric version of BR96, an internalizing MoAb that binds to a highly expressed mutant Lewis Y antigen, abundant on breast, lung, and colon tumors (63) is currently reported to be under active development. However, in the field of drug targeting many investigators are looking for drugs with potencies similar to the phytotoxins for the next generation of drug-MoAb conjugates and are investigating the targeting of highly potent low molecular weight xenobiotic drugs. While too toxic as stand-alone therapies, these drugs theoretically combine the best features of drug and toxin conjugates, having potencies up to 1000-fold higher than clinically approved chemotherapeutic agents without the inherent immunogenicity of the phytotoxins. The promising preclincial data in this field which is now appearing in the literature is summarized below.

Maytansoids are a family of ansa macrolides originally isolated from alcoholic extracts of plants of the Maytenus species (64). Maytansine (May 1a), the lead compound in this series has been characterized as a microtubule poison, which shows antimitotic effects at doses 100-fold lower than vincristine. Maytansine is active over a 50- to 100-fold dose range against P388 leukemia and against a variety of other murine tumors, but failed clinical trials because of a variety of overt toxicities (65). The May derivative (1b) which has similar potency to maytansine in vitro has recently been linked by way of a disulfide bond to a series of internalizing MoAbs: TA.1, an anti-HER2 MoAb, and 5E9, an anti-transferrin MoAb and A7, an anticolon MoAb. The drug conjugates of the general structure Ab~SS-May have molar IC_{50} values of 10^{-10} to 10^{-11}, equivalent to the potency to the parent drug, maytansine, in clonogenic assays on antigen-positive cells (66). A reducible disulfide bond appears to be important for release of the active drug from these conjugates, as conjugates prepared with a noncleavable thioether bond were 200-fold less potent under equivalent experimental conditions. The maytansinoid conjugates with the MoAb 5E9 have recently been shown to prevent the growth of the intraperitoneal A375 human melanoma tumor in SCID mice

without lethality at doses where a mixture of the MoAb and drug itself have no effect (67). The maytansinoid conjugate with the anti-colon MoAb, A7 has been shown to clear much more slowly from blood in mice than the parent maytansine itself, which has a half-life in serum of only 20 minutes. Prolonged serum half-life is expected to be an important advantage for conjugated drugs, particularly if the bond between the drug and the MoAb is serum stable. Improvements in the serum stability of the disulfide linkage in the maytansinoid conjugates are currently being made (66).

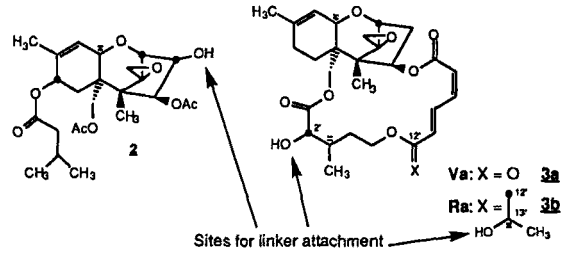


Maytansine: X = H

May-SS-Me: X = $SSCH_3$ **1b**

Site for linker attachment

Trichothecenes - The trichothecene mycotoxins, fungal metabolites of *Fungi imperfecti*, are potent inhibitors of protein synthesis (68). Both simple and macrocyclic tricohothecenes have been identified (the macrocyclic versions being more potent). The macrocyclid trichothecene, Verrucarin A (Va **3a**) has been shown to be at least 100-fold more active than doxorubicin against tumor cell lines in vitro and to have potency similar to *pseudomonas exotoxin* on a molar basis. These drugs were highly active against murine leukemia, but a variety of severe toxicities observed in animal studies and in the one clinical trial of anguidine (a simple trichothecene) in colon cancer have limited their further development as single agents. Preliminary descriptions of conjugates of both simple and macrocyclic trichothecenes have recently been made (69). A hemiglutarate derivative of the simple tricothecene, T-2 (**2**), activated by N-hydroxsuccinimide was attached to an antibody specific for the murine EL-4 thymoma. The conjugate was selectively cytotoxic toward EL-4 cells in vitro and active in an in vivo model as well (70). This T2-MoAb conjugate was believed to be internalized into the lysozomal compartment of the target cells where release of the active drug occurred by hydrolysis. Verrucarin A (Va **3a**) and Roridin A (Ra **3b**), have also been prepared for conjugation to MoAbs. Va has been derivatized in the 2' position with either an N-hydroxy-succinimide ester or a hydrazide and conjugated to NR-LU10, a MoAb that recognizes a pancarcinoma glycoprotein. Data from these studies show that the hydrazide conjugate has similar activity as the drug itself, probably due to release of the drug at the cell surface from this non-internalizing MoAb, while the amide-linked conjugates were 10-fold less active (71). Recent reports suggest that the Ra derivative may be more stable to hydrolysis in serum. The preparation of conjugates from active esters of Ra, prepared by modification at either the 2' or the 13' carbons with a variety of MoAbs is underway, as is the study of the release of the free trichothecene under a variety of experimental conditions (72)



2

Va: X = O **3a**

Ra: X = **3b**

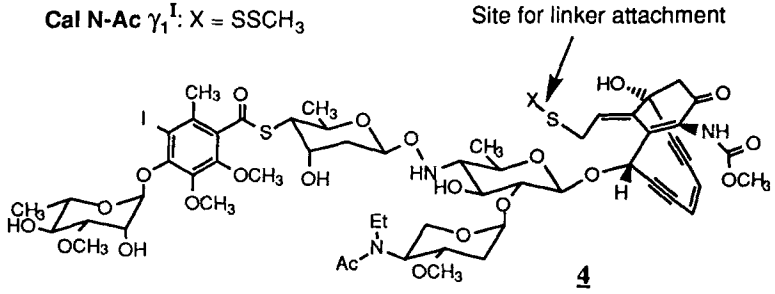
Sites for linker attachment

Enediyne Antibiotics - A third class of potent antitumor antibiotics that have recently been described as MoAb conjugates are the enediynes. These potent microbial products contain a unique bicyclic ring system that is triggered by reduction to generate a biradical which causes DNA strand scission. Four members of this family: neocarzinostatin (NCS) (73,74), calicheamicin (75), esperamicin (76), and dynemicin (77) have been isolated and characterized as potent DNA cleaving agents which are highly cytotoxic in vitro and show antitumor effects in animal models (78). Of this series, only esperimicin is currently in clinical trials as an unconjugated drug.

NCS, the most widely studied enediyene, is not a small molecule but rather is composed of an acidic polypeptide chain, 11 kD, which protects the highly labile bicyclic chromophore (78). Conjugates of NCS with A7, an internalizing murine MoAb against human colon cancer, had excellent activity against xenograft tumors (79). In clinical trials for colorectal and pancreatic cancer the A7-NCS conjugate showed significant antitumor responses which were, however, accompanied by immune responses to even a single dose of A7-NCS in all patients studied (80). No reports have yet appeared on studes with humanized versions of A7, although, several attempts to minimize the immunogenicity of murine A7-NCS conjugates have been reported. Chemical modification of the MoAb with polyethylene glycol (PEG) has been studied and A7-PEG showed an increased circulation time when compared with the parent MoAb A7 and reduced normal organ uptake (81). Local administration of the A7-NCS conjugate has been shown to produce effective control of colonic tumors in murine models and intratumoral injection has been suggested as a less immunogenic route for administration of A7-NCS conjugates (82). Recently, NCS has been linked by disulfide and thioether linkages to Fab fragments of GA-17, a MoAb that binds to an antigen on the surface of human astrocytomas (83). This conjugate is highly active in xenograft models and could potentially be delivered directly into the tumor or cerebral spinal fluid for treatment of malignant gliomas with a minimal opportunity for the development of immunogenicity.

The calicheamicins are small molecule enediyene antibiotics, with a molecular weight of ~1300, which do not have a protein component like NCS. Calicheamicin γ_1^I, the most potent and abundant member of this family is more than 1,000-fold more potent than adriamycin in murine tumor models (84). The extreme potency of the calicheamicins has been attributed to the characteristic sequence selective double-strand breaks in DNA, caused by these compounds (85 ,86). Because of dose-limiting toxicities in animal models, no clinical trials of calicheamicins as single agents have been initiated. Recent studies indicate that some derivatives of the calicheamicins show excellent preclinical activity when conjugated to internalizing MoAbs(87).

A disulfide analog of the calicheamcin derivative, N-acetyl-γ_1^I (4), has been conjugated to both the carbohydrate and lysine residues on the polyepithelial mucin MoAb, CT-M-01, which targets an antigen abundant on breast, ovarian, and colonic carcinomas. The conjugates prepared with both humanized (CDR-grafted) and murine versions of CT-M-01 produced tumor regressions in antigen-positive xenograft tumors without lethality over a four-fold dose range (88, 89). Conjugates of N-acetyl-γ_1^I with humanized MoAbs are currently under development for the treatment of both ovarian cancer and AML.



4

CONCLUSIONS

This review is intended to describe the current status in the development of MoAb-directed effective cancer therapeutics. The tumor killing effects of MoAb-based therapies can result from: natural effector functions, radioisotopes, protein toxins, growth factor neutralization or receptor blockade, sensitization to chemotherapeutic drugs, or potent cytotoxic drugs (5, 6,10). All these approaches require the generation of human/humanized MoAbs with low immunogenicity that will allow multiple dosing. The focus of this review has been the development of non-immunogenic MoAbs and MoAb conjugates of highly potent small molecule cytotoxics. Using a combination of human or humanized antibody and potent, low molecular weight cytotoxic drugs, it should be possible to obtain therapeutic benefit from a MoAb-drug conjugates with limited side effects.

Although preclinical animal model data are impressive, evidence that these therapies will be effective and non-immunogenic clinically has yet to be established.

The first generation human/humanized MoAbs are currently in clinical trials. The current approaches may require further refinement before the true potential of these targeted therapies can be realized in the treatment of major solid tumor types. For example, the penetration of antibody conjugates into solid tumors may need to be optimized through the use of antibody fragments, rather than the use of intact antibodies (90, 91, 92). In addition, the heterogeneity of tumor antigen expression may require the use of bispecific antibodies (93), antibody cocktails, or co-administration of immune modulators to increase antigen expression (94, 95). ADEPT (antibody directed prodrug therapy) may also address problems of tumor penetration and antigenic heterogeneity (96). Research on such novel strategies will proceed while awaiting the first clinical readout on therapies involving human/humanized MoAbs which should be available within the next two years.

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Chapter 26. In Vivo Diagnostics for Alzheimer's Disease Based on the Acetylcholine Transporter

Stanley M. Parsons and Gary A. Rogers
Department of Chemistry and Neuroscience Research Institute
University of California
Santa Barbara, California 93106

Introduction - A number of diseases impact cholinergic nerve terminals. Probably the most important of these to society is Alzheimer's disease. Degeneration of cholinergic terminals in the hippocampus and frontal cortex are among the earliest deficits in this neurodegenerative disease. A sensitive in vivo diagnostic that determines the functional status and/or density of cholinergic nerve terminals in brain might provide a means to detect the onset of Alzheimer's disease before cognitive deficits occur. This capability will be exceedingly important as treatments are developed to stop or reverse the progression of the disease, for patients might remain asymptomatic if the disease were diagnosed in time to initiate prophylactic therapy. Such a diagnostic also would be useful in distinguishing among alternative possible causes of dementia so that appropriate therapy could be initiated.

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) have been used to investigate the function of the cholinergic nervous system in living human brain for about the last 10 years (1). These technologies are described below. The above studies focused on classical cholinergic receptors. Only modest changes in receptor densities have been found for Alzheimer's patients, possibly because most classical cholinergic receptors in degenerating regions are postsynaptic and those that are presynaptic are present at relatively low density. An early diagnostic probably must probe a concentrated target found only in the cholinergic presynapse. The characteristic function of cholinergic nerve terminals, which constitute about 6% of the cortical terminals in mammalian brain (2), is to synthesize and release acetylcholine (ACh). The sodium-dependent high affinity choline uptake transporter in the cytoplasmic membrane (which provides precursor), choline acetyltransferase in the cytoplasm (which synthesizes ACh), and the ACh transporter in the membrane of synaptic vesicles (which mediates storage of ACh for evoked release from the vesicles) are key proteins in the synthesis and release of ACh. The first step (namely high affinity uptake of choline) has been probed with [^{11}C]choline by PET. However, as all cells take up choline, the method is too nonspecific to be useful as an in vivo marker of cholinergic nerve terminals (3).

Among the proteins that carry out presynaptic metabolism of ACh, the ACh transporter (AChT) complex has the most highly developed pharmacology. A potent family of compounds that binds allosterically to the AChT is known. Most of the compounds are relatively easily synthesized and many of them exhibit favorable pharmacokinetics in vivo. Also, the local concentration inside the nerve terminal can be above 1 μM for the AChT complex, which is a very high value. This occurs because there are multiple copies per vesicle and a large number of the vesicles are present in most cholinergic terminals (4). For these reasons, the AChT is a target of choice for the quantitation of cholinergic nerve terminals. This review outlines what we know of the AChT. It also presents the current status of efforts to develop ligands for the AChT that can be used in SPECT or PET to map the density of cholinergic nerve terminals in living human brain.

Spect and Pet - In SPECT and PET a ligand specific to the target is produced that contains a high energy gamma- or positron-emitting isotope, respectively. The labeled compound is injected into the patient, distributed by the circulation and bound by the target in the tissue of interest. In the case of SPECT, the single gamma photon arising from nuclear decay is detected outside of the patient by a scintillant camera. A collimator between the patient and the scintillant establishes the

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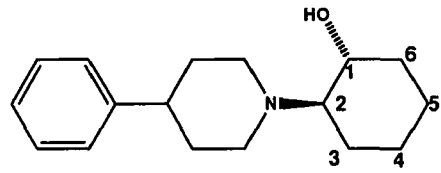
line-of-origin for the decay. In the case of PET, the two gamma photons that are created by annihilation of the positron released by nuclear decay travel in opposite directions and are detected in different camera elements as a coincidence (or near-coincidence) event that establishes the line-of-origin for the decay. Tomographic reconstruction of the decay events produces a three dimensional map of probability for ligand binding in the target tissue, and under favorable conditions the map corresponds to the density of the ligand binding sites. Because of the very high specific activities that such ligands have due to the short half-lives of the isotopes utilized, a good image of binding results from the injection of a very small amount of the ligand. This is critical when probing with a toxic ligand, such as the ligand family to be described below.

The Electric Organ Model - Development of our knowledge about the molecular pharmacology and structure of the ACh storage system has depended largely on a highly purified preparation of synaptic vesicles from the electric organ of the marine ray Torpedo. Because of the hostile or otherwise special environments in which they live, marine animals often have developed an otherwise ordinary characteristic into an extraordinary hypertrophic state in order to adapt. Torpedo produce high-frequency discharges of about 60 volts and 10 amperes that they utilize to stun small fish which they then swallow. They also defend themselves from predators with the discharge (5). More or less ordinary (6,7) cholinergic nerve terminals innervate pancake-like postsynaptic cells called electrocytes that make up most of the electric organ. The electrocytes produce ordinary excitatory potentials of about 60 mV. The prodigious electrical output of the fish is produced by the stacking of about 1000 electrocytes on top of one another in side-by-side columns, much like adjacent stacks of coins (5). This produces a series summation of the excitatory potentials and a parallel summation of the excitatory currents (Figure 1).

Unlike most excitable cells, the electrocytes of the Torpedo are not electrically excitable. The ACh released in the electric organ from synaptic vesicles excites electrocytes chemically by binding to a postsynaptic nicotinic ACh receptor. Because this is the only type of excitation that occurs, cholinergic nerve terminals cover up to 80% of the ventral face of the electrocyte in order to obtain full excitation. The neural density is about equal to that of mammalian brain, but in contrast to brain essentially all of the nerve terminals are cholinergic (6). Thus, the electric organ is a superb, simplified source of the components of the cholinergic synapse. Several milligrams (protein) of highly purified synaptic vesicles containing about 300 pmol AChT per mg protein can be isolated readily from this source. The purified vesicles actively accumulate exogenous ACh when supplied with MgATP.

MOLECULAR PHARMACOLOGY AND STRUCTURE OF ACH STORAGE

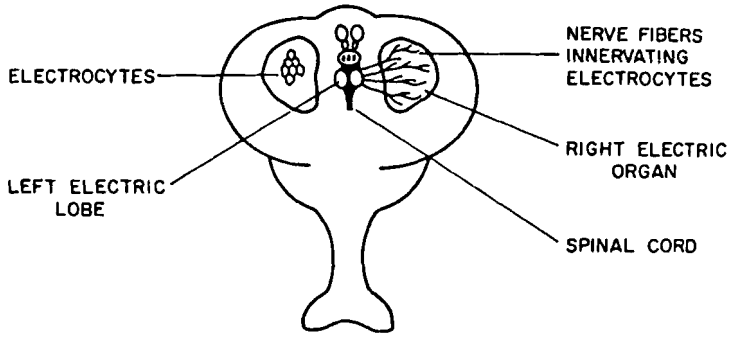
The structure of the ACh storage system is partially known. It is composed of the vacuolar ATPase that pumps protons into the vesicle and a separate AChT complex that exchanges one or more luminal protons for extravesicular (cytoplasmic) ACh. The AChT is inhibited by the drug vesamicol [trans-2-(4-phenylpiperidino)cyclohexanol] (1), which acts noncompetitively at low nanomolar concentrations by binding to an allosteric site (the vesamicol receptor, VR) on the cytoplasmic surface of the AChT (8). The cytoplasmic localization of the VR distinguishes it from the extracellular localization of other receptors typically studied by SPECT and PET. The (-)-enantiomer of vesamicol, which has the 1-R,2-R absolute configuration, is much more effective than the (+)-enantiomer (4,9). A quantitative kinetics model of ligand binding, active transport of ACh and transport inhibition by vesamicol has been developed for the electric organ vesicles (10).



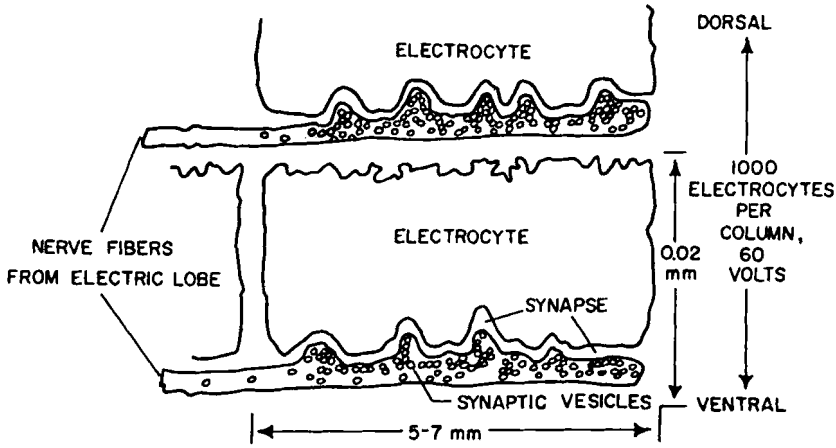
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Recently the AChT was identified by photoaffinity labeling (11), and the vesamicol receptor (VR) was identified by purification of the detergent-solubilized VR through use of a binding assay for [^{3}H]vesamicol (12). Unexpectedly, both of the identified species are 50 to 250 kDa in SDS PAGE. The very broad M_r distribution and other physicochemical properties of the AChT and the VR are

proteoglycan-like (12,13). Proteoglycans are composed of protein that is bound covalently to a special class of linearly polymerized and highly negatively charged sugars called glycosaminoglycan.



DORSAL VIEW OF TORPEDO CALIFORNICA SHOWING CUTAWAY OF THE BRAIN AND ELECTRIC ORGANS



SCHEMATIC VIEW OF VERTICAL SECTION OF ELECTROCYTE INNERVATION

Figure 1. The Torpedo electric ray and its electric organ. The top half of the figure gives a dorsal view of the fish with the brain, the nerve bundles that control the right electric organ, and both electric organs exposed. The bottom half gives a vertical section of the right electric organ that shows the electrocytes with the cholinergic innervation.

Most proteoglycans are in extracellular matrix, but it has been known since 1982 that Torpedo vesicles contain a large amount of proteoglycan, the function of which was unknown. The similarities in the properties of the AChT and the VR that are present even after denaturation in SDS, which include association with a glycosaminoglycan epitope called SV1, suggest that the AChT and the VR are in the same polypeptide. Whether the AChT-VR is associated with the proteoglycan covalently or noncovalently through an interaction that is extraordinarily resistant to denaturation is not known (Figure 2).

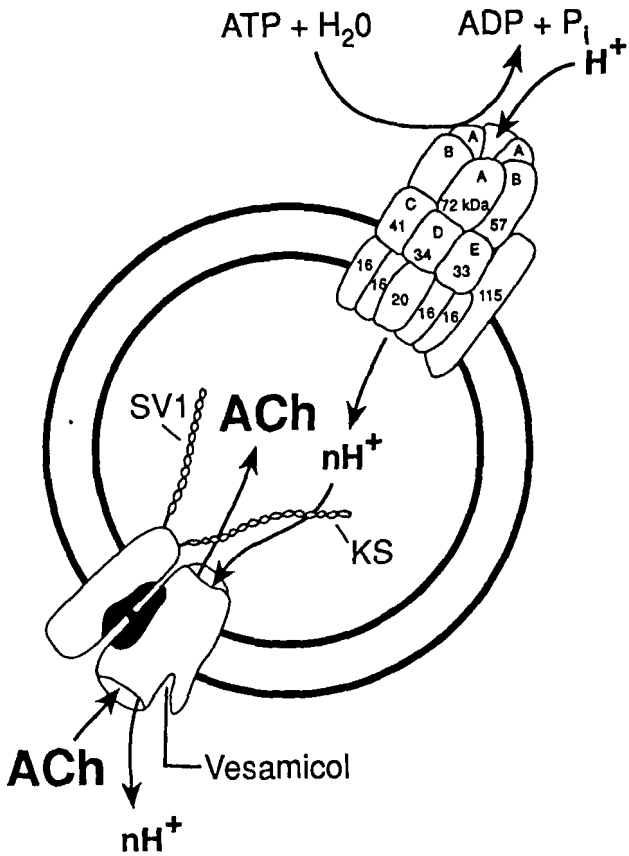


Figure 2 The acetylcholine transport system of the cholinergic synaptic vesicle. The vacuolar ATPase is to the top right and the AChT-VR is to the bottom left. The ATPase pumps protons into the vesicle and the AChT exchanges n protons for one molecule of ACh. Vesamicol binds to an allosteric site on the cytoplasmic surface of the AChT, which is linked in an unknown way (via the narrow bridge) to proteoglycan that carries the SV1 epitope on the keratan sulfate (KS) type of glycosaminoglycan in the lumen of the vesicle.

New analogs of vesamicol were synthesized and evaluated for their abilities to inhibit active transport of ACh by the purified electric organ vesicles (14). This structure-activity study revealed that the alcohol and protonated tertiary amine of the compound are pharmacophores. Substituents placed on the cyclohexanol ring in the cis-4 and trans-5 positions that promote the diaxial conformation of the alcohol and amino groups yield tightly binding analogs. Evaluation of the potencies of analogs by competition against the binding of [^{3}H]vesamicol to electric organ vesicles revealed that the benzovesamicol series that has been used in SPECT and PET (see below) exhibits dissociation constants in the low picomolar range (15).

Physiological and Behavioral Effects of Vesamicol - The structure-activity data obtained with synaptic vesicles of the electric organ support the development of ligands for cholinergic nerve terminals in mammalian brain, providing that Torpedo and mammals share the same pharmacology of ACh storage. The following evidence makes it likely that the VRs of Torpedo and mammals are similar. Vesamicol blocks storage (and thus release) of newly synthesized ACh in every semi-intact cholinergic preparation examined (fourteen types to date), including rat and mouse brain

preparations (16,17). Used in moderate, saturating doses of about 1 μM, the drug is specific in its physiological action on cholinergic nerve terminals. The drug is effective in vivo after stereotaxic, localized injection into the mammalian brain. Vesamicol enantioselectively and potently decreases the release of ACh measured by intracerebral microdialysis of rat (18,19). Injected into the mesopontine tegmentum of rats, it inhibits antinociception otherwise produced by administration of nicotine (20). Vesamicol injected into the lateral cerebroventricle reduces the pressor and bradycardic responses of the spontaneously hypertensive rat to physostigmine, which is an ACh esterase inhibitor (21). The in vivo results support the hypothesis that vesamicol binds to the AChT-VR in the functional mammalian brain.

Vesamicol administered IP is lethal at sufficient doses, and the convulsions that precede death indicate that the drug penetrates the blood-brain barrier (14). Vesamicol also must penetrate the cytoplasmic membrane of the nerve terminal in order to reach the VR. The tertiary amino pharmacophore defined in the structure-activity work with purified electric organ vesicles not only is critical to binding at the VR, it also probably is critical to reaching the VR in vivo. Almost surely the unprotonated form of the drug mediates permeation through the membrane barriers.

Binding of [^{3}H]Vesamicol to Brain Preparations - In this and related in vitro techniques, specific binding of a radiolabeled drug is defined as the difference between the total binding measured in the absence of any other drug and the nonspecific binding measured in the presence of a saturating concentration of a "specific" drug, which often is merely a 100-fold excess of the non-radiolabeled form of the same drug. Binding of [^{3}H]vesamicol to slices of rat brain has been studied by quantitative autoradiography (22-24). Specific binding occurs with a dissociation constant of about 11 to 22 nM, which is similar to the value for electric organ vesicles under similar conditions. Binding correlates well with other markers of the cholinergic nerve terminal, such as ACh, choline acetyltransferase (ChAT) enzyme activity, and ChAT-like immunoreactivity. However, lesions of cholinergic projections to the hippocampus and frontal cortex result in the fractional loss of only about half as much of the specific vesamicol binding as ChAT. A similar result has been obtained using membranes prepared from a homogenate of the cerebral cortex of rats and a filter binding assay for [^{3}H]vesamicol (25).

Membranes obtained from homogenates of human brain specifically bind [^{3}H]vesamicol with about the same affinity and B_{max} as the membranes from rat brain and with about the same affinity and 0.3% of the B_{max} as the vesicles from Torpedo electric organ (26). However, the amount of specific binding in membranes from the frontal cortex of Alzheimer's patients decreases only 17-35%, compared to a 54% decrease in the amount of ChAT. A synaptic vesicle fraction prepared from human caudate nucleus exhibited enrichment in the specific binding of [^{3}H]vesamicol relative to other membrane fractions and a dissociation constant of 20 nM (25). However in contrast to the desired result, specific binding of [^{3}H]vesamicol increases about 40% in crude membranes obtained from Alzheimer's patients compared to controls.

The studies of both rat and human brain preparations clearly demonstrate that changes in the levels of specific binding of vesamicol do not correlate with loss of cholinergic nerve terminals. This divergence occurs even in normal aging, as the ChAT level in normal aged human brain decreases about 30% compared to young brain whereas specific binding of vesamicol increases about 40% (26). These binding studies are consistent with the presence of a second noncholinergic binding site for [^{3}H]vesamicol that is distributed similarly to cholinergic nerve terminals. A ligand suitable to visualization of the VR in complex organs possibly must be more selective than vesamicol. Also, it is possible that the above results obtained with brain preparations are misleading as to the specificity of vesamicol. It is not uncommon that in vitro ligand binding does not mirror the in vivo results when techniques allow a direct comparison.

VESAMICOL ANALOGS SUITABLE FOR SPECT AND PET

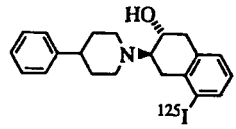
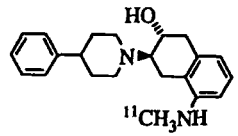
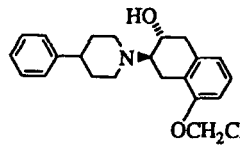
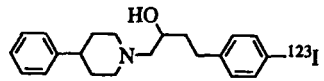
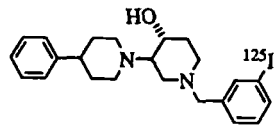
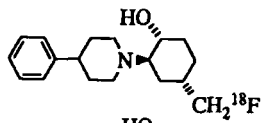
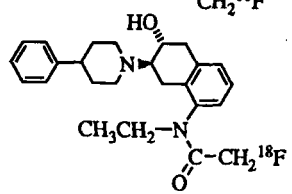
The structures of published vesamicol analogs that incorporate gamma or positron-emitting isotopes are shown in Table 1. Most of the analogs have higher affinity for the VR in electric organ vesicles than vesamicol does due to substitution at the 4 position of the cyclohexanol ring. They thus might be more selective for the VR in mammalian brain than vesamicol. However, the extent of

specific binding exhibited by a lethal ligand is more difficult to assess in vivo than in vitro. This is because the required saturating, 100-fold excess of ligand terminates the experiment. Thus, a complete block of the specific binding of vesamicol analogs investigated by SPECT and PET often cannot be achieved, and the extent of the selectivity of vesamicol-based SPECT and PET ligands for cholinergic nerve terminals has been difficult to determine.

4-[^{125}I]iodobenzovesamicol (>20,000 Ci/mmol, **2**) was synthesized (27,28) as a model for the ^{123}I-labeled species that would emit a higher energy gamma photon suitable for SPECT. The enantiomers were resolved and characterized for their distribution in mouse brain. As determined by measuring the radioactivity in excised regions after injection into the tail vein, the (-)-enantiomer binds nearly irreversibly to striatum and cortex at 27- and 11-fold higher levels, respectively, than to cerebellum. These ratios are consistent with a cholinergic distribution of binding. The near-irreversibility of the binding presumably is due to very high affinity of the ligand and high local concentrations of the VR. About 1% of the injected dose is extracted by brain and very little metabolism was found. Prior injection of a near-lethal dose of vesamicol blocks much of the binding, and the (+)-enantiomer of the iodinated drug is much less selective for cholinergic regions. Other ^{125}I-labeled analogs also were made, but they are not promising (28). 4-[^{11}C]methylaminobenzovesamicol (700 Ci/mmol, **3**) was synthesized (29) and the enantiomers were resolved. This compound is suitable for PET. Binding in mouse brain studied by the same technique as used for the iodinated drug (i.e., not by PET) was found to occur in the rank order striatum>cortex>hippocampus>hypothalamus>cerebellum, which is consistent with cholinergic innervation (30). About 1.5% of the injected dose is extracted by brain and no metabolites were found. The effect of the lipophilicity of labeled aminobenzovesamicol derivatives on uptake by brain was studied (31). The PET analog (-)-4-[^{18}F]fluoroethoxybenzovesamicol (>2000 Ci/mmol, **4**) has been synthesized and preliminarily characterized in vivo (32). Greater than 2% of the injected dose is extracted by brain in mouse, and no metabolites were found.

A number of analogs that are isomerically more simple than the benzovesamicol series, due to opening of the cyclohexanol ring, yet retain the additional hydrophobic bulk (relative to vesamicol) that induces a large increase in affinity have been synthesized (33). The new compounds were analyzed for potency by competition against binding of [^{3}H]vesamicol to the electric organ vesicles. One of these compounds (HIPP, **5**) was produced in [^{125}I] - and [^{123}I]-labeled forms and studied in rat brain by autoradiography and in monkey brain by SPECT, respectively (34). This series of analogs does not appear to be potent or specific enough to be the basis of a useful diagnostic for cholinergic nerve terminals. Derivatives of azavesamicols, which incorporated a wide range of substituents at aza sites corresponding to the 4- or 5-position of the cyclohexanol ring of vesamicol, were synthesized and the potencies were evaluated with electric organ vesicles (35). N-benzyl substituted 4-azavesamicol [1-benzyl-4-hydroxy-3-(4-phenylpiperidinyl)piperidine] is as potent as vesamicol, and 4-azavesamicol has been named trozamicol. Although the potent enantiomers are dextrorotary, it is possible that the absolute configuration of (+)-trozamicol is the same as that of (-)-vesamicol. The analog meta-[^{125}I]iodobenzyltrozamicol ([^{125}I]MIBT) (1500 Ci/mmol, **6**) was synthesized, the enantiomers were resolved, and the distributions of the enantiomers in rat brain slices were determined by autoradiography after intravenous injection (36). About 1% of the injected dose was extracted by the brain. The distribution of (+)-[^{125}I]MIBT correlated moderately well with that of other cholinergic markers after 3 hours of in vivo wash out of nonspecifically bound drug. Particularly high levels of binding were observed in the amygdala, striatum, nucleus accumbens, olfactory tubercle, and the 5th and 7th cranial nerves. (-)-[^{125}I]MIBT did not yield a cholinergic distribution. Co-injection of 4-aminobenzovesamicol with (+)-[^{125}I]MIBT blocked much of the binding, with more blocking occurring in the regions highly innervated by cholinergic neurons. The analog 4-aminobenzovesamicol, which has a dissociation constant of 6.5 pM with electric organ vesicles (15), is the most potent analog of vesamicol known. Co-injection of haloperidol, which is a sigma receptor ligand, partially blocked "specific" binding in the less cholinergic regions. It was concluded that the binding distribution of (+)-[^{125}I]MIBT qualitatively reflects cholinergic innervation, but a fraction of the ligand in cortex and cerebellum binds to sigma receptors. There is preliminary evidence from filter-based ligand binding studies with human cortical membranes and haloperidol that **5** binds to the sigma receptor only somewhat more weakly than to the VR (37). Other N-substituted 4-phenylpiperidines are known to bind to the sigma receptor (38).

Table 1. Vesamicol Analogs Under Investigation as Diagnostics

Compound	Structure	Emmission[a]
2		γ
3		β^+
4		β^+
5		γ
6		γ
7		β^+
8		β^+

[a] β^+ is the positron and γ is the gamma photon.

Cis-4-[^{18}F]fluoromethylvesamicol (>2000 Ci/mmol, 7) was evaluated in living rats and monkeys by PET using a new small animal technique for the rats (39). In rats 20-30% of the tracer is rapidly extracted from blood into the brain, where it is irreversibly bound. In monkeys it is preferentially bound in brain regions known to contain high densities of cholinergic nerve terminals. Preinjection of vesamicol substantially blocked binding in both rats and monkeys. The data obtained during the time course of the binding require a kinetics model containing at least three

compartments in order to achieve a good fit. The compartments are thought to correspond to drug in plasma, free drug in brain, and bound drug in brain. The relevant rate constants for movement among the compartments were determined by regression analysis. Similar results have been obtained in rats for the analog 4-N-ethyl-4-N-[^{18}F]fluoroacetamidobenzovesamicol (>2000 Ci/mmol, 8), except that the extraction into brain was only about 2% (40,41). The lesser extraction of this compound compared to cis-4-[^{18}F]fluoromethylvesamicol is due to both a slower rate of transport across the blood-brain barrier and a slower rate of binding to the VR. The analog 4-N-[^{18}F]fluoroacetamidobenzovesamicol also was examined but it is rapidly metabolized.

CONCLUSIONS

Synaptic vesicles isolated from the electric organ reliably indicate the affinity of vesamicol analogs for the VR in mammalian brain. Thus at the level of ACh storage, the marine model indeed appears merely to be a hypertrophic state of otherwise typical cholinergic innervation. Most vesamicol analogs are metabolically stable in mammals and many of them distribute similarly as expected for a probe of the cholinergic nerve terminal. However, binding of the analogs to sigma receptor complicates the quantitation of cholinergic nerve terminal density in brain. Because of their very high affinities for the VR, the benzovesamicols appear not to exhibit this problem, but compelling evidence of specificity for the VR based on lesion studies has not been presented. Also, the low extraction by brain and irreversibility of binding exhibited by the benzovesamicols are not desirable characteristics for imaging by SPECT and PET. Low extraction requires administration of higher doses of radiation. Irreversibility of binding favors binding by those terminals that are perfused first by the circulation, and modeling of the kinetics of ligand binding and quantitation of the density of binding sites are compromised. Nevertheless, despite the various shortcomings of the available analogs, the utility of the vesamicol family for visualization of cholinergic nerve terminals has been demonstrated. Thus, this approach to diagnosis of Alzheimer's disease has great promise, but better ligands are needed. Because of its modularity, the chemical structure of vesamicol is amenable to extensive structure-activity analysis. Within some clearly delineated constraints, the VR allows considerable variation in the structures of tightly binding analogs. Extraction by the brain, selectivity for the VR and the kinetics of ligand binding per se all probably can be optimized through further structure-activity work. Notwithstanding their current shortcomings, analogs of vesamicol are being tested in humans for diagnosis of Alzheimer's disease by SPECT and PET.

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Chapter 27. The Development and Current Status of Glycoconjugate Vaccines

William Egan
Center for Biologics Evaluation and Research
Food and Drug Administration
8800 Rockville Pike, Bethesda, MD 20892

Introduction - *Haemophilus influenzae*, a Gram-negative bacterium, is the cause of a variety of childhood diseases, including meningitis and epiglottitis. Prior to the introduction of *H. influenzae* vaccines, there were approximately 12,000 cases of invasive *H. influenzae* disease each year in the U.S. (1,2) with accompanying mortality and morbidity rates of approximately 5 and 25%, respectively (3). The major sequelae from *H. influenzae* disease, in terms of numbers and consequences, are mental retardation and loss of hearing (4). The mortality and morbidity associated with *Haemophilus* disease, the increased use of infant day care (increasing the number of susceptible contacts), and the emergence of bacterial strains that are antibiotic resistant (to ampicillin and chloramphenicol), have provided the impetus for disease prevention, through vaccination (5). The historical and conceptual development of vaccines against *H. influenzae*, their current status, and the extension of these vaccine principles to other diseases are the subjects of this review.

Protection against *H. influenzae* disease - *H. influenzae* meningitis is a disease of infants and small children, with the peak incidence of disease occurring before the age of 1 year; as was shown by Fothergill and Wright sixty years ago (6), and confirmed on numerous occasions since (7), the incidence of disease is inversely related to the levels of serum antibodies that are *H. influenzae* specific. The immunity that is observed in older children (> 5 years) and adults is most likely the result either of antibodies formed during periods of asymptomatic *H. influenzae* carriage or of cross-reacting antibodies formed against a variety of organisms that are common to the naso-pharygeal or gastro-intestinal tracts, such as the K100 serotype of *Escherichia coli* (7, 8).

In 1931, Pittman (currently a Scientist Emeritus at the FDA) reported (9) that invasive disease from *H. influenzae* was caused by "encapsulated" organisms, and that of the six encapsulated serotypes (designated by the letters "a" through "f"), the overwhelming majority of disease was associated with only one serotype, namely, type b (see Figure 1 for a schematic rendering of Gram-negative bacterial structure). These findings, related to the association of disease with bacterial encapsulation and serotype specificity, paralleled those of others with *Streptococcus pneumoniae*, a Gram-positive organism. Chemically, the capsules of the six *H.influenzae* serotypes, like those from the pneumococci, are polysaccharides [the composition of a bacterial capsule, as a carbohydrate, was first shown by Heidelberger and Avery (10)].

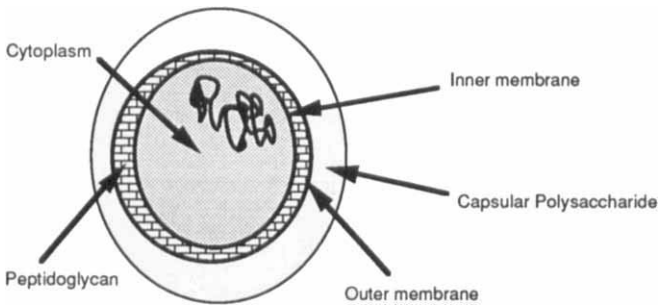


Figure 1. Schematic representation of a Gram-negative bacterium; of the identified structures, only the capsule is optional.

The role of the bacterial capsule as a virulence factor in the disease process, although not understood in its molecular detail, is probably best described as one of shielding the bacterium from the otherwise protective action of the alternate complement proteins (11, 12). The reason for the

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inhibition of the complement proteins by the type b capsule, as opposed to, for example, the type d capsule, is not obvious from naive considerations of chemical structure (see Table 1); nor is virulence simply associated with the quantity of capsular polysaccharide that is elaborated by the organism, since, for example, type d bacteria elaborate as much capsular polysaccharide as do type b bacteria. The serum antibodies that are associated with host resistance and protection are those that are directed against the bacterial capsule (7) and activate the classical complement proteins (12); this holds true for resistance to other invasive, bacterial diseases, such as those caused by the pneumococci or meningococci.

Table 1. Repeating unit structure of the *Haemophilus influenzae* capsular polysaccharides (13).

Type	Structure
a	→4) - β - D - Glc *p* (1→4) D- ribitol - 5- (PO_4 →
b	→3) - β - D - Rib*f* - (1 → 1) D - ribitol - 5- (PO_4 →
c	→4) - β - D - GlcNAc*p* - (1 →3) - α - D - Gal *p*- 1 - (PO_4 → 3 ↑ R [R = OAc (0.8); R = H (0.2)]
d	→4) - β - D - GLcNAc*p* - (1 →3) - β- D - ManANAc*p*- (1 → 6 ↑ R R = L-serine (.41) L-alanine (.41) L-threonine (.13)
e	→3) - β - D - GLcNAc*p*- (1 →4) - β- D - ManANAc*p*- (1 →
e'	→3) - β - D - GLcNAc*p* - (1 →4) - β- D - ManANAc*p*- (1 → 3 ↑ 2 - β - D - fructose
f	→3) - β - D - GalNAc*p* - (1 →4) - α - D - Gal NAc*p*- 1 - (PO_4 → 3 ↑ OAc

The Development of Polysaccharide-based Vaccines - Shortly after the chemical composition of the pneumococcal capsules had been established, Schiemann and Casper (14) demonstrated, in mice, that a purified pneumococcal capsular polysaccharide could stimulate the production of antibodies that were protective against challenge by the homologous organism; three years later, Francis and Tillet (15) reported similar results in man. The definitive demonstration of the *clinical* efficacy of a pneumococcal capsular polysaccharide vaccine required the large, well-controlled, trial conducted for the U.S. government by MacLeod and colleagues in 1945 (16). The emerging availability of antibiotics after World War II resulted, however, in the cancellation of plans to develop pneumococcal vaccines commercially for the general public (17). At that time, most physicians believed that it was easier, and preferable, to treat pneumococcal disease with antibiotics rather than to prevent it from arising through vaccination. This view was not altogether unwarranted, since more than 20 capsular types (out of 83) are involved in pneumococcal disease and the then-available vaccine was only hexavalent; penicillin was effective against all the capsular types (17). For the reasons presented above concerning *H. influenzae* disease, this emphasis on disease treatment rather than prevention has changed. Two 23-valent pneumococcal polysaccharide vaccines are currently in use in the U.S.; they are not, however, targeted to an infant population.

A large-scale efficacy trial with a purified *H. influenzae* type b (Hib) capsular polysaccharide vaccine was conducted in Finland in 1974 (18). Two major conclusions emerged from that trial: first, in children between the ages of 18 and 72 months, the Hib vaccine induced moderately high levels of serum antibodies that were directed against the type b capsule, and the presence of these antibodies correlated with protection against disease; second, infants and toddlers, between the ages of 3 and 17 months, were not protected against disease and the vaccine did not induce significant levels of antibodies against the type b capsule in this age group. These results, which were mirrored in other Hib polysaccharide vaccine trials, were both satisfying and disappointing: satisfying in that older children were protected against disease; disappointing in that the vaccine

was not effective in the younger children, for whom it was the most needed (*ca.* 75% of *H. influenzae* disease occurs in children under 18 months of age).

Why did the Hib polysaccharide vaccine fail in infants and young children? The answer to this question must ultimately be presented in terms of the detailed development of the immune system and in the distinctions that exist among immunogens (19, 20). For this review, however, a simplified explanation can be given. There are, broadly speaking, two distict classes of antigens -- thymus independent (generally termed T-cell independent) and thymus dependent (generally termed T-cell dependent). Polysaccharides are T-cell independent antigens while proteins are T-cell dependent antigens. The ability, in humans, to respond to T-cell dependent antigens is present at birth, whereas the ability to respond to T-cell independent antigens is not; this latter immuno-competence develops slowly, and is functionally present only in the older child. One is therefore faced with an apparently non-resolvable problem -- while it has been shown that protection against *H. influenzae* type b disease is associated with the presence of serum anticapsular antibodies, it has also been shown that infants do not respond well to capsular polysaccharide vaccines.

Development of *H. influenzae* glyco-conjugate vaccines - The conceptual development for the resolution of the above problem, and the eventual introduction of efficacious vaccines for use in infants against *H. influenzae* type b disease, trace back to the late 1920's and the immunochemical studies of Goebel and Avery (21, 22). They prepared protein conjugates of the pneumococcal type III capsular polysaccharide and demonstrated that: (i) in rabbits, antibodies against the polysaccharide were induced by the conjugate, whereas all attempts to produce antibodies with the pure polysaccharide were unsuccessful; (ii) the vaccinated rabbits were protected against challenge by type III organisms, but not other pneumococcal serotypes; (iii) mice could be passively protected with the rabbit antisera.

Fifty years later, Schneerson, Barrera, Sutton, and Robbins (23) reported the preparation and immunogenicity (in mice and rabbits) of protein conjugates of the *H. influenzae* type b polysaccharide. Their study showed: (i) that the immunogenicity of the type b polysaccharide was increased (relative to the polysaccharide alone) when coupled to a protein carrier, but not when coupled to another polysaccharide (the pneumococcal type III polysaccharide); (ii) that re-injection of the conjugates gave rise to an increase in the number of responding animals as well as to the levels of induced antibodies; (iii) that the immune system could be "primed" for the conjugate by prior injection of the carrier protein alone; and (iv) that the induced antibodies were bactericidal. Preliminary experiments showed, moreover, that bactericidal antibodies could be induced in young primates by the conjugate vaccine (23). These findings, like those of Goebel and Avery so many years before, were indicative of a T-cell dependent response and furnished the experimental and conceptual basis for the development of a vaccine against invasive *H. influenzae* disease for use in infants. There are now four licensed type b *H. influenzae* glycoconjugate vaccines in the United States. A discussion of these vaccines, including several aspects of their synthesis, follows.

The U.S.-licensed Hib conjugate vaccines - The four glycoconjugate vaccines that are licensed in the United States for the prevention of Hib disease are listed in Table 2. Clinical studies relating to the efficacy of these vaccines have been carried out and are presented in references 24- 29.

PRP-D (sold under the trade name, ProHIBit) was the first U.S.-licensed (December, 1987) glycoconjugate vaccine. The vaccine was indicated for the routine immunization of children between the ages of 18 months and 5 years. Although PRP-D behaves like a T-cell dependent antigen, it is poorly immunogenic in infants 2 - 6 months of age (30); the chemical basis for this lack of immunogenicity has not been elucidated. The vaccine was prepared using the strategy described by Robbins and co-workers (23), with diphtheria toxoid used as the carrier protein. Since the Hib polysaccharide does not contain functionalities that can be easily used for coupling to a protein, the polysaccharide was activated with cyanogen bromide; the nature of the CNBr activated species has not been determined. The activated polysaccharide was coupled to diphtheria toxoid that had been derivatized with the bifunctional, "spacer" molecule, adipic acid dihydrazide.

The PRP-CRM197 vaccine (trade name HibTITER) is compositionally unique among the four vaccines listed in Table 2: it uses an Hib oligosaccharide, as opposed to polysaccharide, and the oligosaccharide is coupled directly to the protein without the use of a spacer molecule. The vaccine uses a non-toxic mutant of diphtheria toxin (CRM197; CRM = cross-reacting material) as the carrier protein. The Hib oligosaccharides are generated by a limited treatment of the polysaccharide with sodium periodate, forming oligosaccharides that have reactive aldehydic functionalities at each

end. The oligosaccharides are linked directly to the lysine amino functionalities of CRM197 *via* a reductive amination. Acid-catalyzed hydrolysis of the conjugate vaccine liberates a unique amino acid, hydroxyethyl lysine, the presence of which provides a proof for the covalent attachment of polysaccharide to protein, and its quantitation provides a measure of the extent of coupling. The vaccine was licensed in April, 1990 for use in children above the age of 15 months, and in December, 1990 for use in infants at age 2 months.

Table 2. U.S. licensed *H. influenzae* type b polysaccharide (PRP)[§]-- protein conjugate vaccines and several of their characteristics.

Vaccine	Manufacturer	Carrier Protein	Oligo or Polysaccharide
PRP-D	Connaught Laboratories	Diptheria Toxin	Polysaccharide
PRP-CRM_{197}	Praxis/Lederle	Non-toxic mutant of Diptheria toxin	Oligosaccharide
PRP-OMPC	Merck, Sharp, & Dohme	Outer membrane protein complex from Group B, *NMeningitidis*	Polysaccharide
PRP-T	Merieux/Connaught	Tetanus Toxoid	Polysaccharide

[§]"PRP" is the commonly-used discriptor for the type b polysacchride, which is Poly Ribose-Ribitol-Phosphate (*cf.* Table 1).

The Hib-OMPC vaccine (trade name PedvaxHIB) was licensed in 1990 for use in infants starting at age 2 months. The conjugation scheme (31) is depicted in Scheme I. The vaccine uses the outer membrane protein complex (OMPC) from Group B *N. meningitidis* bacteria as the carrier protein. The polysaccharide to protein spacer derives in part from the polysaccharide and in part from the protein (see Scheme I). Hydrolysis of the resultant glycoconjugate results in the liberation of the unique amino acid, hydroxymethyl homocysteine. The formation of this amino acid establishes the covalent attachment of the polysaccharide to the protein and its quantitation provides a measure of the extent of coupling. The vaccine is immunologically distinct among the Hib glycoconjugate vaccines in that it behaves, in part, like a T-cell independent antigen (eliciting a measurable immune response after the first dose), and in part like a T-cell dependent antigen (in that the immune response can be boosted).

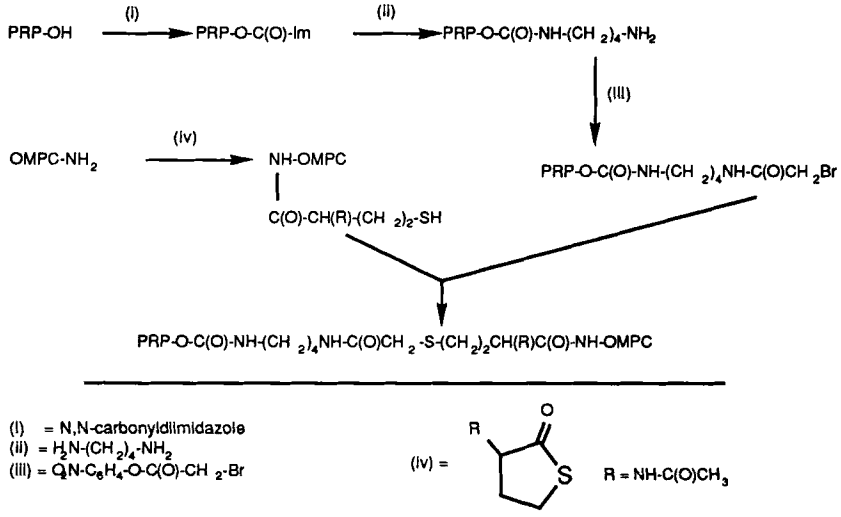
The most recent *Haemophilus* b conjugate vaccine is PRP-T (trade name ActHIB), licensed in March, 1993 for use in infants at age 2 months. It is prepared from a cyanogen bromide-activated polysaccharide that is coupled to tetanus toxoid *via* an adipic acid dihydrazide spacer. Unlike PRP-D, the adipic acid dihydrazide is reacted with the cyanogen bromide activated polysaccharide, not the protein, and this material is then coupled to the tetanus toxoid.

Four different strategies have been shown to be effective for the development of conjugate vaccines against Hib disease; indeed, three of these strategies have lead to effective infant vaccines. There are, however, no clear, guiding principles (in the sense of indicating the best way to produce the next vaccine) that the development of these vaccines have suggested. The immunological distinctions that do exist among them, however, are certainly reflections of the unique aspects of the strategies and chemistries that were employed.

The Impact of the *H. influenzae* Glycoconjugate Vaccines on Public Health - Since the introduction of the conjugate vaccines in 1990, the incidence of Hib disease in the U.S. has decreased dramatically. Between 1980 and 1988, the annual incidence of Hib meningitis in children under the age of 5 years was *ca.* 20/100,000 and for children under 1 year of age, *ca.* 55/100,000; in 1991, the first year following the introduction of the infant vaccines, the annual incidence of meningitis in children under 5 had dropped to *ca.* 5/100,000 and that for infants less than 1 year of age, *ca.* 12/100,000 (32). A similar trend was noted in a study of U.S. army children (33). When figures become available for 1992, it will not be the least surprising to see even lower incidences of Hib disease. Although the efficacy of a vaccine cannot be readily established by monitoring the incidence of disease relative to historical controls (because other factors may also be varying), the observed relationship between the decrease in disease and the introduction of the Hib conjugates (see Figure 3 of reference 33) is certainly consistent with the efficacy that had been shown in the individual trials (24 - 29). Furthermore, it is gratifying to see that the conjugate vaccines are

immunogenic (28) in groups that are at especially high risk for Hib disease such as Native Americans (the incidence of disease in these groups is *ca.* 5 - 10 times that of the U.S. population in general).

Scheme I.



VARIABLES IN THE PRODUCTION OF GLYCOCONJUGATE VACCINES

Four of the major chemical-immunological variables to be considered for the development of glycoconjugate vaccines are listed in the box below; these variables are discussed in this section.

1. Size of the oligo- or poly-saccharide
2. Hapten loading.
2. Protein -- saccharide attachment chemistry.
3. Choice of the carrier protein.

Saccharide Size and Hapten Loading - The dependence of the immunological response on oligosaccharide size (*i.e.*, degree of polymerization, DP) has been investigated by several groups (34 - 38). These studies of oligosaccharide-based conjugates do not point to a single, optimal DP (or DP range); rather, they point to a variability of the optimum, reflecting the unique character of each vaccine construct. It has been suggested (39) that the minimum oligosaccharide size is that one which will fill the antibody combining site; this corresponds to approximately six sugar residues (40). The optimal length may be considerably longer than that required to fill the combining site if, for example, the antibody is directed against a geometry that forms only with increased chain length, a "conformational epitope" (41 - 44). A study of conjugates made with high and low molecular weight *Salmonella typhi* Vi polysaccharides found that the high molecular weight polysaccharide conjugates were more immunogenic than the low molecular weight polysaccharide conjugates (45); the generality of this finding with polysaccharides needs to be explored further. Indeed, it is not yet clear whether the better vaccine strategy is to use an oligosaccharide or a polysaccharide.

Several of the above studies indicated that increased "hapten loading" (the number of oligosaccharide equivalents coupled to the carrier protein) correlates with increased immunogenicity (34, 35). Again, it will be of interest to determine whether this is a general trend, and to further determine the point at which immunogenicity begins to drop off. The extent of protein derivatization on immunogenicity has been studied for hapten-protein and peptide-protein conjugates (46,47); these studies indicated that for moderately sized proteins, peptide to protein (or hapten to protein) ratios between 5:1 and 20:1 resulted in good responses, but that the response fell off at higher ratios.

Nature of Saccharide -Protein Linkage - From the viewpoint of the number of potential strategies for linking saccharides to proteins, this might well be the most complicated variable. The saccharide may be linked to the protein at only one end, for example, at the reducing sugar (as in Fig. 2a) or at both ends, forming networks of varying complexity (Fig. 2b). The saccharide might also be linked to the protein at one or more internal sites and, again, be attached to either one (Fig. 2c) or several proteins (Fig. 2d). Moreover, the saccharides and proteins can be linked directly or through spacer molecules and the saccharide can be attached to the protein in a site specific or random fashion. It is not yet known whether specific attachment sites on the protein are to be preferred or avoided for purposes of protein processing by the T-cells (20).

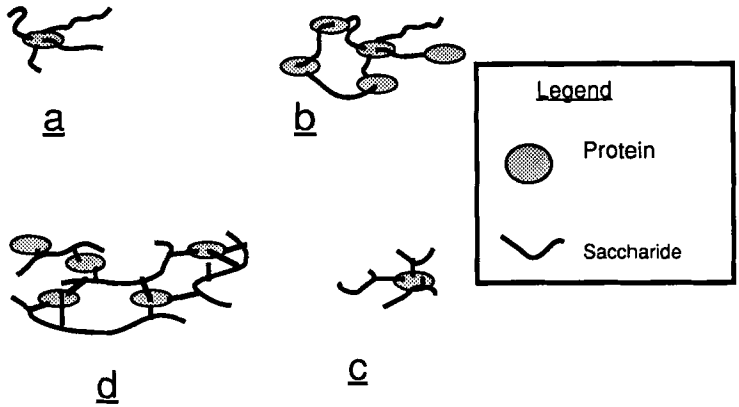


Figure 2. Possible configurations for glycoconjugate vaccine assemblies.

Carrier Protein - There are a number of factors to be considered in the choice of a carrier protein, including: cost and availability, purity, safety, protein and conjugate solubility and stability, the number and type of available reactive sites (and the potential for side-reactions within the protein), and immunological utility. A carrier protein can be immunologically useful in various ways. A protein for which the immune system has been primed (or will be primed simultaneously), such as the diphtheria and tetanus toxoids, can be used as a carrier, with the expectation of obtaining boosted levels of antibody against the polysaccharide. Alternatively, a protein that has not previously been seen by the immune system, but against which an incidental immunological response might be beneficial (as examples, cholera or pseudomonas toxins or the OMPC from Group B *N. meningitidis*) can be used. In evaluating candidate carrier proteins, the potential for damage from inducing antibodies that cross-react with host tissue must be considered; for example, the cell wall M-proteins from the Group A β-haemolytic streptococci would be poor choices for carrier proteins because of the potential for cross-reactions between these proteins and host cardiac tissue. Finally, for reasons such as cost, availability, or chemical strategy, a neutral protein (one that the immune system will see only in the vaccine) could be used; there are no examples of the use of a neutral protein in vaccines for human use, although such proteins have been used in animal studies

Defining and monitoring the relevant variables and designing for robust responses - In order to produce and ensure a consistent glycoconjugate vaccine, the immunologically relevant chemical variables must be defined carefully and monitored accurately. Although obvious in principle, defining and monitoring the relevant variables may be difficult to achieve, particularly when the variables are interrelated or the conjugation chemistry is not well defined. Considerable care needs to be taken in the design of this chemistry in order to be able to define and monitor the relevant variables.

The immunological response to a variation in a physical-chemical parameter, *e.g.*, one of those mentioned above, should follow a schematic curve as in Figure 3. For example, consider the immunological response as a function of the degree of polysaccharide activation or derivatization. In the limit of no derivatization, there will be no immunological response. With increasing derivatization, there will be an increasing amount of attachment of polysaccharide to protein and a concomitant enhancement of the desired response. At some point the polysaccharide will become

over-derivatized and the targetted immune response will decline eventually decreasing to zero as the derivatized polysaccharide resembles less and less the starting antigen. To the extent possible, the derivatization chemistry should be targeted to a middle value, avoiding the downward edges. By first carefully defining the response curve and then targeting the middle of the curve, the variations in vaccine chemistry that will inadvertently occur during manufacture, will result in the least change in immunological response.

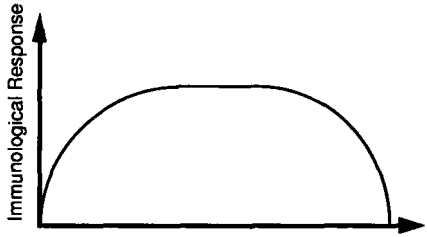


Figure 3. Generalized immune response as a function of change in chemical variable (schematic).

GLYCOCONJUGATES AND THE FUTURE

Additional Glycoconjugate Vaccines. Based, in part, on the success of the *H. influenzae* vaccines, a number of additional glycoconjugate vaccines are now in various stages of development; a representative selection of targeted pathogens for glycoconjugate vaccine development is given below in Table 4. Of the vaccine candidates in Table 4, that for Group B *Neisseria meningitidis* deserves special comment. Unlike the majority of polysaccharide antigens (*vide supra*), the Group B polysaccharide (an α-2->8 linked homopolymer of sialic acid) is not immunogenic in adults. It has been postulated (see refs. 48 and 49 and references cited therein) that this lack of immunogenicity is due to the immunological similarity, or even identity, between the Group B polysaccharide and various host antigens, such as the sialic acid oligomers found on gangliosides; a potential danger with a Group B conjugate vaccine, therefore, is that it could break immunological tolerance (20) and induce self-antibodies. Efforts have been made to develop Group B vaccines based on cross-reacting antigens, either a modified Group B antigen (48) or cross-reacting *E. coli* (48), viz., the K92 serotype (58).

Table 4. Glycoconjugate Vaccine Development

§Pathogenic Organism	Associated Disease/Syndrome§	Reference*
Group B, *Neisseria meningitidis*	meningitis, septicemia	48, 49
Cryptococcus neoformans	meningitis (in immunocompromised individuals)	50
Salmonella spp.	acute gastroenteritis, septicemia	51
Shigella spp.	acute gastroenteritis	52
Staphylococcus aureus	septicemia	53
Group B *Streptococci*	septicemia, meningitis	54
Streptococcus pneumoniae	otitis medis, pneumonia	55
Vibrio cholera	cholera	56

§ The diseases or disease syndromes listed with the various pathogens are not all-inclusive; for more complete descriptions, see reference 57.

* The list of references for the various glycoconjugate vaccine candidates is illustrative, not all-inclusive.

Additional Vaccine-related Issues - Several other vaccine-related issues are now being explored that need to be considered for current glycoconjugate vaccine design programs. These areas include improved means of vaccine delivery, the use of combination vaccines, and the development of adjuvants for boosting and directing the immune response.

With the recent increase in the number of childhood vaccines (there are currently nine recommended pediatric vaccines) and the potential addition of others, *e.g.*, an *S. pneumoniae* vaccine, the need for combination vaccines is increasingly coming to the forefront, both to minimize trauma to the infant and to increase vaccine compliance by decreasing the number of required visits to the healthcare provider (59). Combining vaccines is, however, not always without problems. In addition to issues relating to timing (age at which a vaccine series can start and the interval between the recommended doses), the interactions between vaccine components, as they affect stability and immunogenicity, must be carefully explored. As an example, when inactivated polio vaccine (IPV) was first combined with DTP, it was found that the polio potency decreased because of the preservative in the DTP component (60).

In addition to combination vaccines, alternative vaccine delivery systems, such as the lactide-glycolide microspheres, are now being widely investigated (61, 62). Microspheres can serve a number of roles. For example, by altering microsphere composition, the rate of antigen release can be controlled, offering, in principle, a means to deliver several doses of vaccine in one injection. The microspheres can also be used to deliver vaccines orally or intra-nasally; such altered delivery could result in the elaboration of mucosal (IgA) as well as the humoral (IgG) responses. The microspheres also appear to have an adjuvant activity.

New adjuvants (63, 64) are now being explored to enhance and direct the immune response. In addition to the generally well-recognized role of elevating the immune response and serving as a depot for the immunogen, adjuvants can also direct the immune response; that is, direct the response to a particular immunoglobin class or sub-class, for example, IgG_1 as opposed to IgG_2.

The extent to which particular glycoconjugate vaccines can be used interchangeably also remains to be fully explored. For example, if PedvaxHIB is used for the primary vaccination, can ProHIBiT be used for the booster? As the number of glycoconjugate vaccines and glycoconjugate vaccine manufacturers increases, this aspect of immunization will become increasingly important.

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Chapter 28. HUMAN GENE THERAPY

Gerard J. McGarrity and Yawen Chiang
Genetic Therapy, Inc.
Gaithersburg, MD 20878

Introduction - Significant progress has been made in recent years to treat human diseases by the insertion of human genes. At this writing, the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) has reviewed and approved 52 different clinical protocols in human gene transfer/gene therapy. These are summarized in Table 1. In addition to RAC review, the Food and Drug Administration (FDA) must also approve gene transfer protocols. These, however, are not reviewed in public meetings. In addition to these protocols in the United States, gene transfer trials are underway in Italy, France, the United Kingdom , Germany and the Peoples Republic of China. More countries will certainly join this list in the near future.

Some of the human clinical trials approved in the United States are for the purposes of gene marking; i.e., to determine the fate of genetically marked cells through the use of a gene conferring antibiotic resistance to the neomycin analogue G418. In this presentation, the term gene transfer denotes the use of foreign genes inserted into human cells for purposes of either therapy or marking. The term gene therapy refers to clinical trials performed solely for therapeutic objectives.

A number of comprehensive reviews on gene therapy have recently been published (1-2). The Journal, *Human Gene Therapy*, regularly publishes clinical protocols that have been reviewed by the RAC. Readers can consult the Journal for more complete descriptions of specific protocols. In this presentation, references will be made to clinical protocols that have either been reviewed or approved by the NIH RAC. The reason for this is these are public meetings, and protocols are available. FDA review is confidential and reference to the protocols approved by the RAC do not necessarily mean they have been approved by the FDA, or that the trials have been initiated.

The most significant developments in human gene transfer in recent years have been related to vector design and the potential use of viral vectors other than retroviral as well as non-viral vectors; design of appropriate safety studies; studies in animal model systems for the treatment of cancer; use in infectious and genetic diseases; and the continued acceleration of clinical trials.

Table 1. Gene Transfer Clinical Trials Approved by the NIH RAC as of May 1993

Total Approved Trials:	52	
gene therapy:	30	
gene marking:	22	
cancer trials:	19	
genetic diseases:	9	cystic fibrosis, 5 adenosine deaminase deficiency, 1 familial hypercholesterolemia, 1 Gaucher disease, 2
infectious diseases:	3	AIDS related
liver dysfunction:	1	

VECTOR SYSTEMS

Retroviral Vectors - The majority of clinical trials continue to utilize retroviral vectors. The retroviral vectors currently in clinical trials for human gene transfer have been derived from members of the Oncovirinae subfamily of the Retrovidiae family. Retroviral and other viral vector systems for gene transfer have been recently reviewed (3).

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The genome of murine retroviruses is simple. Coding sequences are flanked by long terminal repeats (LTRs) that contain the control elements for transcription, packaging, polyadenylation (poly A) signals as well as the sequences that are required for replication and integration. Three different types of proteins are encoded: 1) (gag), structural proteins; 2) (pol), enzymes including reverse transcriptase, integrase (int), and a protease; and 3) (env) which encode for major envelope proteins gp70 and p15e(4).

Retroviruses are diploid with each virion containing two full length RNA molecules. The receptor for ecotropic viruses has been characterized (5), and normally functions as a cationic ion transporter (6,7). A human analogue has been identified which has 87.6% homology to the murine receptor (5). The human receptor for murine amphotropic virus has not been identified, although the putative receptor gene has been localized to the pericentromeric region of human chromosome 8 (7).

Retroviral vectors which are producted in packaging cells (3) are characterized by their ability to transduce proliferating cells as opposed to non-proliferating cells and to integrate in a random fashion into their host cell genome (8). The factors that might modulate the integration site of retroviruses have been discussed (8). Two major concerns for the use of retroviral vectors in clinical trials are the potential for retroviral vectors to produce insertional mutagenesis and to generate replication competent viruses (RCR) (9). The probability for these retroviral vectors to cause mutagenesis and perhaps carcinogenesis either through activation of oncogenes or inactivation of tumor suppressor genes is thought to be low, on the order of background levels(10).

Table 2. Titers Generated from N2 in Mixed Packaging Lines

Packaging Cell Line	Day post-transfection	S+L-	Vector titer (CFU/ml)[a] 3T3	Mv 1 Lu
ψ2/PA317	16	+	2.5×10^6	1.5×10^5
GPE86/PA317	13	-	5.5×10^6	2.4×10^5
PGE86/PA317	17	+	1.1×10^6	2.9×10^5
ψcre/PA317	18	-	8.7×10^3	5.0×10^4
ψ2/PA12	11	+	1.3×10^4	2.7×10^4
GPE86/PA12	12	+	3.8×10^4	4.3×10^4
ψ2/Ycrip	17	-	8.1×10^3	0
GPE86/ψcrip	19	-	6.0×10^3	0
ψcre/ψcrip	17	-	5.0×10^2	9.0×10^1
PA317	15	-	1.8×10^3	5.5×10^3
PA12	12	-	1.0×10^3	5.6×10^3
ψcrip	11	-	0	0
ψcre	12	-	0	0
GPE86	11	-	1.7×10^2	0
ψ2	15	-	7.0×10^2	0
PA12	___[b]	+	5.4×10^5	6.5×10^5
ψ2	___[b]	-	2.4×10^6	1.1×10^1

Packaging line cocultures are given as ecotropic/amphotropic pairs.
[a]CFU represent G418 resistant colony forming units. Murine 3T3 cells were selected in 600 μg/ml G418 and Mv 1 Lu mink fibroblasts (ATCC CCL64) were selected in 800 μg/ml.
[b]Virus stock obtained from transfected and selected clones that produce high titers.
Table modified from ref. 9.

Retroviral producer cells will vary in their ability to generate RCR. This will be influenced by the degree of homology between the helper and vector genome (1,3,4). In some systems, the vector genome is mutated to change the gag start codon to a stop codon (3). With such a design, two recombinational events and a point mutation would be required to generate an RCR. A second approach to minimize the potential for RCR generation is the use of a "split-packaging" producer cell line. In this design, the coding sequences of the helper genome are separated into two distinct units. Several such systems have been described (4,9). Three recombinational events would be necessary to generate RCR in split packaging systems. However, the conversion of a producing cell system from a standard to a split packaging cell will not, a priori, lessen the possibility of an RCR

"breakout". Table 2 lists the potential for breakout of RCR in some packaging cell systems. Breakouts may be due to either homologous or non-homologous recombinational events (11). One description of a breakout has been described and a review of RCR as a safety concern in human gene therapy has been published (12). Interestingly, in the reported breakout event, simple biological assays, consisting of inoculating the sample into a direct S+L- assay detected the RCR.

Some of safety concerns of retroviral gene transfer in human clinical trials have been reviewed (12). Retroviral vectors in current use are designed to be replication incompetent. In addition to safety studies cited above involving deliberate inoculation of RCR in non-human primates, one study demonstrated the potential of RCR to cause lymphomas in these animal hosts (13). In this study, severely immunosuppressed Rhesus monkeys received autologous transplants of retrovirally transduced bone marrow cells. The immunosuppression consisted of radiation that ablated both lymphoid and myeloid cells as well as 5-fluorouracil. Three of eight animals that received these transplants developed lymphomas. The genome of the tumor cells contained 50-100 proviral insertions, but significantly, no vector. It was known that the transplanted cells contained RCR in the range of 10^4-10^6/ml in the presence of 10^8-10^9/ml vector, but based on earlier studies(14), it was assumed that the monkeys could overcome this level of RCR. This study is significant in that it demonstrates that RCR, if present in overwhelming numbers, can overcome a crippled immune system to produce disease.

The results of the breakout of RCR and the development of tumors in immunosuppressed monkeys clearly demonstrate the need for reliable and sensitive assays to detect RCR. Assays for RCR can consist of inoculation of specimens from production lots into PG-4 cells as well as inoculation into 3T3, *Mus dunni* and IMR-90 cells for amplification prior to inoculation into PG4 cells for S+L- assays. In addition, aliquots of cells from each product lot are propagated for a three week period beyond harvest and assayed by S+L- for the presence of RCR.

Considerable work has been performed on retroviral design. Vectors often contain a marker gene such as for neomycin resistance (neo^r) to facilitate characterization and titering in addition to the therapeutic gene of interest. The effect of one gene on the other is unknown, (position effect), but can theoretically influence overall gene expression. Similarly, whether to place the primary gene of interest under the control of the LTR or an internal promoter is typically decided on a case by case basis. Multiple constructs in which the therapeutic gene is expressed alternately by the LTR and an internal secondary promoter are made, and multiple clones of each are assayed for titer and gene expression. It can be difficult to determine if differences between various constructs are due to differences in promoters or to clonal variation unless specific and controlled studies are performed.

Adenoviral Vectors - Replication incompetent adenoviral vectors are being used in human gene therapy clinical trials for cystic fibrosis (CF). Typically, these vectors have been rendered replication incompetent by deletions in E1A-E1B and E3 regions of the adenoviral genome. Adenoviral vectors offer advantages for some applications of gene therapy. These include the ability of these vectors to transduce non-replicating cells, making them attractive to target airway epithelial or liver cells. Adenoviral vectors can be propagated to high titer after purification, in the range of 10^{10}-10^{11}/ ml. Adenoviral vector expression can be achieved without integration of the vector DNA into the host genome, thereby eliminating the potential of insertional mutagenesis. However, there have been reports to suggest that adenoviruses can establish latent infections in human lymphocytes and macrophages. A 675 base pair sequence in E1A was detected in lungs of adult smokers and non-smokers (15). The sequence was detected in lung tissue of cigarette smokers more frequently ($p<0.002$) than in non-smokers. E1A sequences were detected more frequently than E3/19K sequences. All specimens in this study utilized material from patients undergoing resection for lung cancer.

Adenoviral viral vectors have been reviewed (3, 16). The adenovirus genome consists of a linear DNA molecule of approximately 36 kb with inverted terminal repeats (ITR). Typically, adenovirus infections kill susceptible cells, although not by traditional cell lysis. Adenovirus types 2 and 5 have been the most widely studied sources of vector. These serotypes of subgenus C have not been associated with serious human disease. Millions of individuals have received adenoviral vaccines. Combinations of recombinant adeno and hepatitis vaccines have been prepared (17). Adenoviruses encode for an estimated 2,700 polypeptides. Most of these are associated with structural components, the capsid, capsid-associated or the nucleoprotein core (3, 16).

In the construction of adenoviral vectors, mutants with deletions in the E1A and E1B regions have been generated in producer cell lines (18); however, vectors with deletions in the E3 region have also been described (19). The human cell line, 293, has been used most often as a producer line for adenoviral vectors(20). At high multiplicities of infection, the E1 region apparently was not crucial for replication (2). The major safety concerns associated with adenoviral vectors are:

the possibility of generating replication competent adenoviruses (RCA) by rescue or recombination and the potential of an immune response upon repeated inoculations. These questions will be answered by clinical trials. The NIH RAC has currently approved five clinical trials that employ adenoviral vectors, but presently all involve a single administration of vector.

The utility of adenoviral vectors will have to be determined by risk assessments, safety testing, and by clinical experience involving dose escalation and repeat administration. However, an impressive amount of in vitro and in vivo preclinical data have been generated using a variety of genes and model systems. Many of these have involved airway epithelial cells. These vectors have been used to successfully transfer the human CF transmembrane regulator (CFTR) gene in vitro and in vivo (21). The human CFTR transcripts were detectable for up to six weeks and the CFTR protein was detectable for 11-14 days. These data would suggest that the CFTR gene and its promoter are constitutive in nature and apparently do not need to be highly regulated. Retroviral vectors and plasmids could also transfer the CF gene (19,22). Adenoviral vectors also transferred the alpha-1 antitrypsin gene to the lung epithelium of cotton rats (23). Adenoviral vectors have been used to successfully transfect other tissues (24).

A variation of using adenoviral vectors involves the combination of adenoviral capsid and either a polylysine-transferrin or polylysine antibody complex. The DNA encoding for the transgene is attached to the complex. The adenovirus moiety functions to allow the complex to escape degradation in the endosome. This system has resulted in transfection of airway epithelial cells in vivo.

Other Viral Vectors - Adeno-associated viruses (AAV) have been proposed as vectors for human trials. These viruses are part of the dependoviruses and are among the smallest and structurally simplest DNA viruses(3). AAV is defective and requires coinfection with an unassociated helper virus for replication. Its attraction resides in AAV's ability to specifically integrate into a small region of human chromosome 19q (25). The mechanism of site specific insertion is unclear. The challenges in successful vector design for AAV include: developing an effective packaging cell system and maintenance of site-specific integration with final vector constructs. The growth of AAV in human T and B cells has been described (26).

The use of Herpes viruses as gene vectors is presently problematic. They are quite large, 150 kb and the switch from latent to lytic infections is not fully understood. Herpes viruses have been proposed as vector systems (27). Herpes vectors have been used to introduce genes to post-mitotic neurons. However, despite the attractions offered by this system, much has to be achieved to develop a standardized and reliable vector system. Even replication-incompetent herpes viruses can kill cells.

Non-Viral Vectors - The major nonviral methods of introducing DNA into cells at an efficiency that might be practical for clinical protocols include use of liposomes, receptor mediated complexes, and direct inoculation of DNA. One clinical study now in progress utilizes introduction via liposome, and an application has been approved by the NIH RAC to use lipofectin to introduce an IGF-1 to brain tumors. No results from this study have yet been disclosed. However, much work has been reported on the use of liposomes as a vehicle for intracellular delivery (28).

Direct inoculation of DNA can result in cell transfection (29) and this technique seems to be particularly promising for muscle cells. This technique has also been proposed for vaccination with microbiological agents.

Receptor-mediated gene transfer can be used for delivery to specific tissues. Most work has been done with asialoglycoprotein which targets preferentially to liver (30). A potential problem with this technique is that endocytic vesicles formed during this process typically end up in lysosomes, where the endosomes are degraded (2). One alternative to this procedure is to employ components of adenoviral vectors which can circumnavigate this problem, as discussed above.

PRECLINICAL AND CLINICAL STUDIES

Cancers - Different approaches have been attempted for gene therapy of human cancer. Ex vivo treatments involve the removal of cells from patients. Types of cells treated with this type approach have included lymphocytes, bone marrow or tumor cells, and expansion of the cells with transduction of all or part of the cells during ex vivo growth. Alternately, vectors or vector- producer cells can be directly administered in vivo or in situ. To date, transfected genes have included neo^r both for marking protocols and in combination with genes for therapeutic purposes. This latter group has included IL-2, IL-4 , GM-CSF, IFN-γ, TNF, IGF-1, MDR, p53, antisense k-ras, and histocompatibility genes. In addition, the thymidine kinase gene from herpes simplex virus is being used as a suicide vector, described below.

The use of ex vivo vs in vivo/in situ gene therapy approaches has implications for commercial production and the number of potential recipients of gene therapy. Ex vivo treatments are patient specific, at this time requiring the growth of patients' cells in the laboratory. This type of approach will require more extensive, and presumably more expensive treatments associated with the introduction of the vector.

The use of in vivo/in situ gene therapy approaches will typically involve the administration of the gene directly to patients without extensive extra manipulations specifically for gene therapy. Specific administrations will vary, but may include those made during surgery, handling of bone marrow, bronchoscopy, aerosolization, or other procedures used for conventional administration of therapeutics.

In addition to classic administration of vectors, one clinical trial involves the direct implantation of retroviral producer cells directly into the bed of glioblastoma multiforme (31, 32). The murine producer cells generate retroviral vectors that encode for the thymidine kinase (TK) gene from herpes simplex (the vector also encodes for the neo^r gene). In this model, tumor cells from mouse, rat, or human produced a lower tumor incidence when containing tumor cells transduced with this vector, G1TkSvNa, and subsequently treated with ganciclovir (GCV). GCV has a significant reactivity to viral Tk (e.g., herpes or cytomegalovirus). This results in a triphosphate that generates toxic metabolites. Of particular note is that a "bystander effect" exists whereby it is not necessary to transduce all 100% of the cells with the TK gene to completely eliminate the tumor. Apparently, following GCV administration, toxic metabolites are somehow transferred from cells expressing herpes TK genes to surrounding cells (31), perhaps through gap junctions. In addition to efficacy in mouse and rat systems, no toxicity has been observed following inoculation into brains of mice, rats and monkeys.

Other suicide vector approaches have been published. The use of producer cells to achieve in vivo transduction has been described (33). Murine brain cells were transduced with the bacterial gene beta-galactosidase with producer cells. Inoculation of vector alone did not produce significant transduction of surrounding neural tissue.

TK has been used as a suicide vector in animal tumor models. One study investigated the efficacy of tumor removal in a model consisting of B16F10 melanoma cells in C57Bl6 mice (34). The investigators reported successful "suicide" of tumor cells in vitro, and tumor regression in the mice. These "cured" mice, however, developed tumors at the site of inoculation "long after" cessation of GCV treatment. These resultant tumors were excised, and found to contain the TK gene. These findings would suggest that noncycling cells during the time of GCV can serve as a reservoir for subsequent tumor growth. This would suggest additional treatments with GCV may be appropriate.

Other suicide vectors have been studied in which the TK gene derived from varicella-zoster was transcriptionally regulated by either the hepatoma associated alpha-fetoprotein or the liver-associated albumin transcriptional regulatory sequences (35). Therefore, expression of the TK was restricted to cells that contained alpha-fetoprotein or albumin positive cells. In these systems, cells that were positive for both TK and either alpha-fetoprotein or albumin were able to convert the prodrug, 6-methoxypurine arabinonucleoside to the toxic metabolite, adenine arabinonucleoside triphosphate. The investigators speculate on the applicability of this and alternate approaches, such as linking TK expression to transcriptional regulatory sequences of carcinoembryonic antigen for treatment of colorectal tumors (35).

An alternate approach to gene therapy for glioblastomas involves the introduction of an antisense-IGF-1 (28). In animal studies, rat glioma C6 cells produce an abundance of IGF-1. A variety of tumors including astrocytomas, meningiomas, sarcomas, thyroid adenomas, estradiol-regulated and non-regulated breast carcinomas, Wilm's tumor, colon carcinoma, squamous cell carcinoma, small cell lung carcinoma, tetracarcinomas, and hepatocarcinomas had elevated levels of IGF-1. Use of antisense IGF-1 was shown to be effective in formation of both subcutaneous and brain tumors in rats. A clinical protocol has been proposed. Some of the clinical protocols currently underway involve manipulation of the bone marrow, either for marking studies or for therapeutic objective. Usually, the clinical objective is to transduce stem cells. In one protocol, it was demonstrated that relapses were traceable to neo^r marked marrow cells, which underscores the need for more effective purging techniques (36).

In non small lung cell cancer, mutations occur in the p53 tumor suppressor gene or in the k-ras oncogene in the vast majority of patients. Most patients will have either the p53 or the k-ras mutation; a small minority will express both mutations (37). It has been postulated that introduction of either the wild type p53 tumor suppressor or the antisense k-ras might be able to reverse the tumor phenotype in those tumors where the mutated forms are expressed. It is anticipated that the introduction of wild type p53, as the dominant allele will be able to suppress the uncontrolled growth provided by the mutant form. Similarly, the antisense transcripts of k-ras are anticipated to be able to block expression of this oncogene while not interfering with expression of other members of

the ras family such as N and H ras that are required for normal cell function and growth. A clinical protocol has been approved by the RAC to pursue this objective. In preclinical studies to support this protocol, human lung tumor cell lines that express either the mutant form of p53 or activated k-ras were transduced with retroviral vectors for either wild type p53 or antisense k-ras (38). In in vitro studies, the tumor phenotype was reversed. Animal models consisted of inoculation of tumor cells into nude mice, subcutaneously for p53 or directly into the lung for anti-sense k-ras. A reduction in tumor incidence was reported. Whether this approach will be successful in humans will have to determined by clinical trials.

Various investigators have attempted to use genes encoding for different cytokines which have included IL-2, IL-4, TNF, and GM-CSF. Animal models have included both immunogenic and non-immunogenic tumor lines inoculated into mice or rats. Viable or irradiated tumor cells transduced with the cytokine under evaluation have been inoculated, either at the time of tumor inoculation or at a later time. This approach has been used with the B16 melanoma non-immunogenic tumor line (39). Results showed that the challenge site of mice immunized with irradiated cells transduced with GM-CSF exhibited large numbers of eosinophils, monocytes, and lymphocytes. Only lymphocytes were observed at the site of mice immunized with irradiated cells. These results have formed the basis of a clinical protocol utilizing tumor cells transduced with GMCSF that has been approved by the NIH RAC. An alternate approach involves the fusion of a tumor-derived idiotype to GM-CSF (40). Such a fusion protein was shown to be a strong immunogen that induced idiotype antibodies. This approach protected mice that had received the fusion immunogen against the plasmacytome Ag8.653. GM-CSF could produce effective immunity at a dose as little as 5 ng.

Genetic Diseases - At the present time, clinical trials are in progress investigating the potential of gene therapy for four genetic diseases: adenosine deaminase deficiency (ADA), familial hypercholesterolemia, Gauchers disease, and cystic fibrosis. The first three trials utilize retroviral vectors; all five CF trials utilize adenoviral vectors. The proposals to treat Gaucher Disease, an illness characterized by a deficiency in glucocerebrosidase, will transduce bone marrow cells with retroviral vectors carrying the normal gene.

Approaches to treat genetic diseases by gene therapy will vary. Factors that influence the development of these clinical protocols will include:

1. level of gene expression needed to achieve "normalcy";
2. degree of regulation of the target gene;
3. target tissue and its level of proliferation;
4. accessibility to the target tissue;
5. therapeutic alternatives; and,
6. clinical objectives.

The clinical objectives of the protocol should indicate whether the treatment is intended to have a curative effect or to reverse the progression of the disease. The answer to this question may impact on the age of the study population recruited. In certain genetic diseases, it may be easier to cure the disease if children or even newborns are treated. This occurred when two newborns having ADA had their cord blood stem cells transduced with the corrective gene. While many of these questions may also occur in cancer protocols, most cancer gene therapy protocols at this time involve patients with a brief life expectancy.

The target tissues for many genetic diseases may depend on the specific disease tissue requirements. Ectopic tissues should be considered. Bone marrow stem cells can be an attractive site as a target tissue (41). These cells are able to renew themselves and have the ability to differentiate into progeny cells after transplantation to produce a self-perpetuating population. Table 3 lists some genetic diseases that either have been cured or in which symptoms have been significantly reduced following bone marrow transplantation. Other diseases have either been unsuccessful or only partially successful, such as Lesch-Nyhan syndrome (41).

Table 3. Candidate Diseases for Gene Replacement Therapy by Gene Transfer into Human Stem Cells (modified from reference 38)

Disease	Gene Defect
Red Cells	
β-Thalassemia	β-globin
Lymphocytes	
ADA deficiency	ADA
PNP deficiency	PNP
Monocytes and Neutrophils	
Chronic granulomatous disease	Cytochrome b, β-chain
Leukocyte adhesion deficiency	CD18 β-subunit
Lysosomal storage disorders (mostly macrophages)	
Gauchers disease	Glucocerebrosidase
Mucopolysaccharidosis i	α-L-Iduronidase
Niemann-Pick type B	Sphingomyelinase
Metachromatic leukodystrophy	Arylsulfatase A

An alternate approach to bone marrow transplantation following transduction is the use of peripheral blood stem cells (42). This source has been shown to contain hematopoietic progenitors and even possibly stem cells. If this is true, the procedure for stem cell transduction could be greatly simplified. This study showed that the transduction efficiencies of CD-34 enriched peripheral blood specimens was comparable to those obtained with CD-34 bone marrow specimens (42).

Results from large numbers of studies have been published on different tissue types being transduced, either with marker genes or with genes of therapeutic interest in vitro or in animal models. It is beyond the scope of this presentation to offer a complete bibliography. However, normal tissue types have included skin fibroblasts, skeletal and smooth muscle cells, endothelial cells, various lymphoid cells, cells derived from neural tissue, keratinocytes and hepatocytes. All of these could also be considered as potential target cells for certain genetic diseases.

REGULATORY AND PRODUCTION ISSUES

The fact that so many clinical protocols have gone forward would suggest that major obstacles do not exist from a regulatory standpoint. Guidelines in the form of Points to Consider Documents issued separately by the NIH and the FDA are clear in what is required from investigators in the field. These Documents are likely to be updated as more data become available regarding risk assessments, safety requirements for adverse reactions, patient monitoring, and manufacturing requirements. Institutions or companies considering submitting protocols should be aware of regulatory requirements.

A major challenge to commercial organizations in this field will be the capability of providing enough vectors or producer cells to meet the anticipated future demand. At this writing, little more than 130 patients worldwide have received some form of gene therapy. The capability to provide tens of thousands or hundreds of thousands of doses will be required in order for the field to advance and for commercial industries to develop. These requirements will pose a serious challenge to cell biologists and bio-engineers. While a history exists for the mass production of adeno virus vaccines, no such precedent exists for mass producing mouse retroviruses. However significant efforts and progress have been made in methods to increase transduction, titers, concentration, and purification efficiencies (43). In this regard, it is pertinent to note that rapid progress was also made in the successful cultivation of large scale volumes of mammalian cell cultures, both hybridoma cells for the production of monoclonal antibodies and CHO cells for recombinant proteins.

It is expected that the next years will see continued progress in the development of viral and non-viral vectors, packaging cells, methods of delivery, specific disease applications, and methods of large scale production of vectors. Significant challenges will certainly exist, such as who, industry of government, will finance the development of gene therapeutics for diseases that have very low incidence in the population or diseases that predominate in developing countries that may not have the resources to pay full development costs of the technologies. Applications for situations that are not apparent will certainly arise. This possibility was heightened by the recent paper on targeting DNA to hair follicles and the possibility that this could represent a model for gene therapy of the hair growth process (44).

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SECTION VI: TOPICS IN DRUG DESIGN AND DISCOVERY

Editor: Michael C. Venuti
Parnassus Pharmaceuticals, Inc.
Alameda, CA 94501

Chapter 29. 3D Database Searching and Docking Strategies

Christine Humblet and James B. Dunbar, Jr.
Parke-Davis Pharmaceutical Research
Division of Warner-Lambert Company
Ann Arbor, Michigan 48105

Introduction - Traditionally, chemical information systems have relied upon two-dimensional (2D) connectivity tables as a means to represent chemical structures. Graph theory (*i.e.*, subgraph isomorphism algorithms, clique detection methods), sometimes coupled with distance geometry approaches (*e.g.*, difference distance matrix), have and continue to be used for structure, substructure and similarity searching driven by geometric or property-based queries (1-5).

Recent advances in computer technologies and dramatic cost reductions for physical memory and mass storage devices now allow for extensive data storage. Simultaneously, modern workstations have undergone a major expansion in computational power, providing the capability to access the hundreds of thousands of data records needed to search and analyze structural as well as conformational information. As a result, impressive progress in the development of automated methods for generating three-dimensional (3D) structures from traditional 2D chemical diagrams has occurred.

The advent of straightforward 2D to 3D structural database conversions has prompted the simultaneous development of methods to retrieve, classify (or cluster), and analyze structural information, especially as they relate to the interrelationships of structures to biological activities. Recent biological technologies have further emphasized the fundamental value of molecular structures, constitutive building blocks that can clarify drug-receptor associations and help understand biological functions of receptor proteins. As reviewed in this chapter, structural databases can provide invaluable resources to medicinal chemists, offering an expanding range of tools to enhance the drug discovery process, including methodologies in information retrieval, knowledge-based strategies, multiple docking procedures, and *de novo* design approaches.

STRUCTURAL DATABASES AND SIMILARITY SEARCHES

Similarity searches initially involved geometric matching of structural groups, a simple strategy using query patterns based on the atom connectivity of the 2D chemical structures stored in databases. For example, searches are commonly used to verify that a particular chemical structure is indeed novel (*e.g.*, patent or spectral search)(6). Common database searching strategies have been reviewed in the recent literature (1,4,7).

2D Database Searches - Such database searches are global in nature, since the query is meant to identify structures that present total or partial similarity. When dealing with structures that relate to biological activities, however, the database queries need to incorporate geometric features to reflect on the presence of particular structural elements that appear to impact on the biological activity, *i.e.*, the pharmacophore or pharmacophoric moieties. A pharmacophore can thus consist of non-connected atoms spread throughout an active structure. It can also be an ensemble of atom types and their distance relationships, pharmacophore moieties (*e.g.*, presence of a particular heterocyclic moiety, H-bond acceptor or donor, positive or negative charges), or receptor site points. Database searching must therefore provide the flexibility to identify crucial substructural or geometric

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elements. The methods of subgraph isomorphism and distance geometry have attempted to address these needs as amply reviewed in the recent literature (4,5,8).

The majority of the 2D searching methods retrieve only exact query matches, overlooking potential hits (9). Newer methods are currently being developed to overcome this limitation. For example, a two-step approach involving unique subsimilarity screening followed by an approximate maximal common subgraph matching step was shown to find substructures that nearly match (*i.e.*, "fuzzy match") the initial query (9).

2D to 3D Database Conversion - The program MOLPAT was among the first to introduce 3D concepts in database searching (10). Evolving from 2D substructure search systems, the first generation of 3D similarity searching systems were aimed at a structural geometry similarity search based on an input target structure. The database search procedure involved a two-level retrieval algorithm in which the time-consuming geometric search was preceeded by a rapid screening search based on interatomic distance ranges (4,5,8). The atom mapping technique is an example where the degree of resemblance between pairs of 3D structures is calculated from their interatomic distance matrices (8,11,12). The computational efficiency of the method was recently assessed and compared to others (8,11).

The emergence of powerful molecular graphic systems has intensified the desire to expand database retrieval mechanisms beyond 2D molecular connectivities to incorporate 3D properties in the queries. Today, most database retrieval systems have been upgraded to 3D structures as examplified in the recent availability of software tools from various commercial sources such as Cambridge Structural Database (CSD)(13) with the QUEST3D and VISTA programs (14), Chemical Design Ltd with MACCS3D (6), Molecular Design Limited with CHEMDBS3D (15), Tripos Associates Ltd. with UNITY (16), Chemical Abstract System (17), and Fine Chemical Directory (FCD) (6).

The rule-based program, CONCORD (18) is the most popular procedure to convert existing 2D databases (19). Using artificial intelligence methods, an alternative approach has been developed in COBRA (20), a successor to WIZARD. This program decomposes structures into "simple conformational units" and progressively assembles reasonable "subconformations" for each unit. The conformational space of a molecule is then represented as a tree-like structural assembly which lends itself to graph-searching techniques. Like all other knowledge-based systems, this method is limited by the initial dictionary of conformational units considered. Efforts to incorporate distance geometry template generation to address this limitation are anticipated.

3D Conformational Flexibility - An important issue in 3D database system development rests with the ability to rapidly and efficiently address conformational flexibility for 3D structures converted from a 2D connectivity table. At this point, there is no universally accepted solution to this problem. For example, conformational information may be stored explicitly in the database in the form of multiple conformations or implicitly in the form of interatomic distance ranges. In the explicit approach, it becomes necessary to preload multiple conformations into a database. Major difficulties lie in the selection and number of conformations to retain for each structure. Not only does this impact the size of the database and the speed of the search, but also the successful identification of relevant conformations (7). In the implicit approach, the conformational flexibility is inferred by multiple interatomic distance ranges, involving distance geometry algorithms, and is widely illustrated in programs such as ALADDIN (21) or EMBED (22). The use of bounded distance matrices, a combination of distance geometry and graph theory methods, was shown to be an efficient means to address the conformational flexibility while matching pharmacophoric patterns in a 3D database (23). Conformational flexibility may also be taken into account while the database search is being performed. Flexible search queries or "on-the-fly" conformational analyses have been reported (24). The Sybyl UNITY database system illustrates another recent initiative to incorporate newer methods such as a tweak algorithm that allow for conformational expansion (16).

STRATEGIES IN 3D SIMILARITY SEARCHING

In the absence of a receptor structure, 3D databases can be searched indirectly, matching the known 3D structures of various ligands on the basis of 3D similarity. This computerized screening is used to identify structural leads (25).

Grid-based Similarity - As originally introduced by Carbo, molecular similarity between two molecules can be determined from the electron density derived from quantum mechanical calculations (26, 27). The technique has since been extended to use other structural properties

such as electrostatic potentials, electric fields, and shape. In the program ASP, for example, molecules are surrounded by a rectilinear grid; the structural property is evaluated at each grid intersection and integrals are evaluated numerically or by utilizing Gaussian functions rather than grids (28-32). The similarity index resulting from these calculations provides a means to quantitatively relate the molecular similarity to the observed biological activities. In the program MEPCONF, molecular similarity is derived from the molecular electrostatic potential (MEP) mapped on a grid surface that is proportional to the van der Waals surfaces of two molecules (33). Both rigid rotations and translations of one system relative to another and internal conformational expansion of the relative molecules are carried out in an iterative procedure to maximize the similarity. The similarity is measured using the Spearman rank correlation coefficient on pairs of points on the common, coincident, grid computed at each iteration.

Surface and shape similarity - The 3D similarity can also be derived from molecular surfaces or shape analyses. For example, a 3D shape definition has been described by considering points on spheres that surround the molecules (3,36). In this gnomonic projection, 3D molecular properties such as size and electrostatic potentials can be assigned as scalar or vector functions.

In a distinct approach, SPERM, an icosahedral mapping and matching procedure (37) has been used to specify molecular properties surrounding molecules (38, 39). 3D molecular shape is the primary matching parameter and requires an initial superposition of centroids or center of mass for the molecules compared. A 3D space scanning is applied to the molecule being compared to a reference template, leading to a shape similarity score evaluated from the sum of squares of the differences (SSD). Any chosen molecular property such as molecular electrostatic potentials or electron density can then be further quantified using either Carbo (26, 27) or Hodgkin (29) similarity indices.

Another method has been derived from the use of approximate wavefunctions (40). The shape of a molecule is defined by exploring the molecular surface with a helium atom probe. The calculation involves the evaluation of a simple expression for the repulsive interaction between molecule and probe. The similarity index is calculated from the overlap integrals between the orbitals of one molecule and those of the other.

Following an initial atom-matching alignment, the molecular shapes have been used to optimize the alignment (41). A surface comparison is evaluated from all surface dots within a certain distance of the corresponding dots of the mean surface. It has been used in an effort to compare biologically active or inactive compounds belonging to a similar structural type.

Two hashing schemes, one based on connectivity and the other based on internal distances, have been applied to the problem of similarity and clustering of shape data (42).

Shape similarities have traditionally used geometrical representations, but topological representations have also been explored (43). The shape group method has been extended to utilize density domains of molecules analyzed at different electron density values (44). The electron density is calculated by an *ab initio* method. The shape codes generated in the analysis can then be used to provide a similarity measure for two molecules in a topological rather than a geometrical description.

Pharmacophore-based Similarity - In the majority of the 3D similarity studies, a preliminary molecular alignment is necessary. Usually, geometric features derived from pharmacophoric hypotheses have been used to guide manual superimpositions of corresponding molecules, sometimes handled through molecular graphics manipulations. Recent studies illustrate the trend to automate molecular superpositions, building upon the 3D database retrieval techniques to limit biases often introduced by individual users.

In the program SUPER, the correspondence of molecules is achieved through optimum superposition of two molecules using their overlap integrals (45). SUPER is designed to identify the best twenty superpositions by generating a network of grid points at the van der Waals surfaces of the two molecules and assigning united atom potentials to these points. The search for the superposition is carried out by rigid body translations and rotations such that each atom of molecule 1 is eventually paired with each atom in molecule 2. SUPER discards matches of points if the difference between two potentials is above a certain set value. This is also used as a measure of the goodness of fit.

The RECEPS program, extended with a module AUTOFIT, is designed to superimpose two molecules on the basis of hydrogen-bonding patterns (46). This is accomplished by an iterative least squares superposition procedure with minimization of both position and direction vectors, checking that the appropriate hydrogen-bonding heteroatom types are correctly matched. This method is capable of conformational expansion based on systematic rotation of torsion angles, and is therefore subject to the combinatorial explosion problem.

In another approach, ligand structures are used to define potential receptor binding sites, the anchor points (47). Algorithms are then applied to identify binding partners for these anchor points and to position them in 3D space. The ensemble of binding partners constitutes a "pseudo-receptor" model for the ligands compared. YAK, a receptor mapping program, allows the orientation of binding partners according to molecular interactions. This program is used in combination with a database of 110 atom types defined on the basis of their interaction properties.

DISCO, based on a clique detection method, determines the superposition of a series of molecules and proposes pharmacophore hypotheses (48). The location of ligand and receptor site points required in the superposition rule, as well as the selection of multiple conformer sets, are prepared with the program ALADDIN (21). DISCO can search for superposition of a set of molecules, or by relaxing tolerances for a match, providing insight into alternate modes of superpositions.

In an approach that can be extended to receptor mapping, hydropathy has been used to measure similarity based on complementarity (49). Hydropathic character may be examined by generating maps for the ligand (KEY program) or for the receptor (LOCK program). The program LOCKSMITH utilizes the maps generated by the KEY program and searches for orientations and common structural features for a series of molecules.

INTERMOLECULAR DOCKING STUDIES

The availability of 3D structural databases has driven the development of methods to locate and analyze protein cavities. More recently, new algorithms have been described to improve the simulation and evaluation of ligand-binding site intermolecular associations as well as protein-protein interactions.

Binding-site Surface Depiction - In CAVITY, a set of algorithms allows the user to isolate and define the extent of a particular binding cavity (50). Solid modeling techniques are employed to produce a detailed cast of the active site region. This method necessitates the selection of an initial seed point in the binding site. Electrostatic and steric properties can be represented on the cavity surface to assist the graphical analysis of the detailed molecular interactions between ligand and receptor surface.

Other approaches to depict the binding site boundaries use different algorithms and present varying levels of accuracy and computer requirements, but all allow automatic identification and visualization of potential binding sites in proteins (51-53). Once the potential binding sites have been located, other programs can be used to examine ligand docking.

Graph Theoretical Techniques - To determine whether any of a given series of compounds are able, on geometrical grounds, to interact with an active site of interest, Smellie and coworkers (54) applied a bipartite graph representation that takes advantage of the clique detection algorithm of Kuhl (55) associated with a clique filtering method. This docking strategy necessitates the identification of matching sites and can be applied to pharmacophore or binding site queries. Conformational flexibility can be incorporated in the form of upper and lower bound distances and distance constraints can be imposed during the docking procedure. This method holds promise as an efficient first pass tool to prepare large 3D database searches. Also based on graph theoretical techniques, a method was described to convert the surfaces of the two (macro)molecules into graphs (56). Appropriate parts of the two surfaces are then matched on the basis of corresponding graphs or patterns.

Shape Complementarity Methods - The program DOCK represents an early attempt to match "keys" into a known "lock" (57,58). 3D databases are searched for potential ligands that are complementary in 3D shape to a known receptor binding site and scored based on their goodness of fit. DOCK has been applied to a series of 103 inhibitors of chymotrypsin and correctly identified the two best known inhibitor structures (59). The authors also reported the screening of the 57,128 compounds available in the FCD; out of the 23 high DOCK-scored ligands tested, two structures

were found to inhibit chymotrypsin. In a more recent version of the program, DOCK2, better sampling and a more systematic searching of the orientation space have been reported (60). DOCK2 also includes a lattice-based method for evaluating the goodness of fit. These new features have been assessed in a variety of applications. DOCK was recently applied to the crystal structure of *L. casei* thymidylate synthase (TS) using the FCD (61). The TS substrate deoxyuridine monophosphate (dUMP), and several known nucleotide and non-nucleotide inhibitors of the enzyme, were identified and adequately scored by the computational screen. Additional molecules unknown to bind TS (*e.g.*, sulisobenzone), were also retrieved from the database and shown to inhibit the TS enzyme.

An approach using bidimensional surface profile to find molecular shape complementarity reduces the complex 3D surface to a 2D representation called "angular profiles". The observed crystal-bound conformations of kallikreine and bovine pancreatic trypsin inhibitors were reproduced, but it was not the best matching solution, suggesting that this method could be used as an initial step toward a 3D study of the shape complementarity between two molecular surfaces (62).

An alternative approach, patterns of points or webs are generated to represent the two molecular surfaces (63). McLachlan's least squares fitting method (64) is then used to match corresponding webs in the two surfaces. Local complementarity and van der Waals interaction energy are used as filtering criteria to select multiple docking orientations. These are then scored based on electrostatic interaction energy. The authors suggest that this method is amenable to coarse dihedral sampling to address conformational flexibility of the ligand.

Another method that uses similarity in the design of compounds involves determining the electrostatic properties of the ligand in the field of the receptor. If electrostatic characteristics are important, then their determination will allow searches for molecules that have similar charge characteristics. A program, YING, determines the point charges of a ligand bound to a receptor based on maximizing the complementarity of the charges of the ligand and receptor (65). The method uses the van der Waals surface of the ligand, the electrostatic potential of the receptor at that surface, and the partial charges of the ligand atoms. The program then maximizes the charge complementarity while still maintaining any formal charge associated with the ligand. Ligand and receptor atoms are kept fixed during this process.

Metropolis Monte Carlo Method - In the program DISDOCK, a simulated annealing procedure is applied to position and orient the interacting molecules, which are both treated as rigid bodies (66). The distance constraints that are necessary to guide the relative intermolecular orientation are derived from a selection of atom pairs such as a hydrogen donor and its complementary acceptor atom. When applied to a set of serine proteinase complexes, the best results are obtained when known distance constraints are available. The results also point to a dependence on the starting ligand conformation.

In AUTODOCK, a Monte Carlo conformational analysis is combined with rapid energy evaluation using molecular affinity potentials (67). A limited number of rotatable bonds can be randomly varied in the ligand structure. Although this approach does not require preliminary knowledge of the binding site, it appears that the substrate binding modes can be affected by the starting orientation. In an alternative strategy, rigid molecular fragments are docked into the binding site (68). The unique feature of this method is the ability of buried fragments to float to the binding pockets from inside a grid representation of the receptor as opposed to placing the ligand at some arbitrary position well outside of the receptor grid. This is accomplished by a scoring function that measures the average distance to the surface points on the grid. Simulated annealing is then used to complete the simulation.

Another method utilizes a two step procedure. A predocked ligand is submitted to an initial coarse sampling (*i.e.*, rigid body translation and rotation) in positional space with limited conformational exploration. A Metropolis Monte Carlo (69) minimization is then applied with complete freedom of movement of the ligand to optimize the structures obtained from the first step. The receptor is rigid throughout the simulation and the method does not use a grid/surface based representation (70).

Protein-Protein Docking - Although the recent literature emphasizes docking procedures that involve a small molecule ligand binding to a macromolecule, molecular complementarity also applies to protein-protein recognition phenomena. Early attempts in protein-protein docking relied either on electrostatics or geometric description of the molecular surfaces (71).

The DOCK program has been extended to the area of rigid body protein-protein docking (72). In a similar approach, simplified protein models with one sphere per residue are subjected to simulated annealing using a crude energy function where the attractive component is proportional to the interface area (73). The procedure finds clusters of orientations in which a steric fit between the two protein components is achieved over a large contact surface.

In another approach, each protein is reduced to sets of surface and internal volume grid points (74). Molecules are then docked by matching surface grid points. Optimal orientations are those with maximum matching surface points and minimum overlapping volume cubes.

DE NOVO DESIGN

Ligand Structures - The program CAVEAT was among the first to utilize 3D database geometry features to retrieve novel structural templates (75). In a recent application, the CSD was searched to extract structures in which specified bond directionalities were maintained at the correct distances and angles to identify structural peptidomimetic templates. In a related approach, the FOUNDATION database system retrieves molecules based upon matching some minimum subset of elements of the query (76). The query elements include atom position and type, bonded and nonbonded atoms, RMS fit, subsets with an occupancy range, and volume restrictions. By using appropriate atom type definitions, general characteristics such as hydrogen bond donor/acceptor and hydrophobicity can be included in the query. The unique feature in FOUNDATION is the ability to search for any combination of some specified lower limit of the elements that comprise the query. This "fuzzy match" allows the retrieval of molecules that do not fit the entire query. This can be used as starting points in the design of novel ligands, or in the selection of compounds to be tested in an assay. Since molecular fragments can be in the input, searches for bridging elements to link the important elements of a pharmacophore hypothesis can be run. The resulting hit list can be ranked by number of matching atoms, RMS-fit, or steric complementarity if a volume constraint is used.

In the program GROW, peptidic fragments are placed and connected within the confines of the receptor active site (77). The template library contains amino acid fragment units in their multiple low energy conformations. From an initial seed point, all template fragments are evaluated for binding site fit. Scoring is based on a molecular mechanics potential, including a solvation term. The growth can take place without any restrictions or can be directed by the use of several control parameters.

LUDI is a rule-based system that makes use of contact patterns found in the CSD to perform automatic design of novel compounds (78). LUDI looks for hydrogen-bonding interactions with appropriate distance and angle characteristics, lipophilic-aliphatic and lipophilic-aromatic interactions. The fragments employed are small molecules progressively connected to fit a binding site. LUDI is now capable of starting from an existing fragment, allowing incorporation of features extracted from a known ligand. The scoring function takes into account the number and quality of hydrogen bonds and also the contact surface area.

Another method, DELEGATE (within the software package BUILDER), generates an irregular lattice of points within an active site (79). First a DOCK run is performed to place a number of appropriate molecules within the active site. This collection of molecules is used to construct the irregular lattice, fusing the atoms of the DOCK structures with correct distance and angles into a composite structure that fits the active site. Additional minimization is required to regularize the structure. Electrostatic, hydrogen bonding, and shape complementarity characteristics can be accomodated. This lattice can also be used manually to view the disconnected structures and prune/connect atoms to form a composite structure.

The database system CLIX (80) utilizes a series of GRID (81) runs to determine where various probes would have favorable interactions with a target receptor. This produces an ensemble of possible sites of interaction. The database is comprised of structures from the CSD. CLIX searches for at least three coincident favorable points of interaction between the grid and the ligand with appropriate steric fit. The fits are scored based on the sum of the interaction energies.

GenStar is designed to grow tetrahedral atoms into an active site (82). The input controls include the heavy atoms of interest at the active site, a seed point (either from the enzyme or from a known inhibitor), and maximum number of atoms required in the compound to be generated. A "closeness grid" is calculated around the seed point to check for steric contacts. A scoring scheme similar to that used in DOCK includes the capability to detect potential hydrogen bonding sites.

LEGEND builds a structure sequentially, starting from randomly selected atom types positioned with random torsion angles (83). Intermolecular interactions are neglected during the atom generation process. A candidate atom is selected automatically if it is not bumping either the enzyme or any previous atoms in the growing candidate molecule. After a structure is complete, charges are assigned to all atoms and the structure is energy minimized in the active site. From the many structures generated by LEGEND, a separate post-processing program, LORE, may be used to select the more interesting structures for graphical analysis.

In preparation for site-directed drug design, the CSD has been used to retrieve aliphatic fragments and their respective bond properties (84-86). A new algorithm to create diverse, irregular, and physically reasonable 3D linear atomic chains or molecular graphs has been described (87). These can be used to generate structural templates for joining up regions in an active site (87).

Protein Structures - The use of structural databases in similarity or commonality searching of protein sequences and structures has been an area of extensive research during the past decade. Applications include prediction of structure from sequence (homology- or knowledge-based), design or engineering of proteins, and 3D model building for ligand design. Recent research has focused on recognizing the structural information available in known structures or substructures. Knowledge-based systems have been developed to access, compile, and analyze protein structure properties such as residue features (*e.g.*, side-chain rotamers), secondary structure (*e.g.*, turn motifs), and tertiary folds (88-90).

The similarity searching methods commonly applied to chemical structures are now finding their first applications in the field of protein structures. For example, a subgraph isomorphism algorithm based on a clique detection procedure has been applied to locate structural patterns in pairs of proteins (91).

EMERGING TOPICS

Clustering Methods - The speed at which 2D structures are being converted into 3D structures has led to an explosion of structural data records based on available databases such as the FCD, CAS, or other proprietary databases. This has led to the need to select a subset of 3D structures to be considered in a database query. Cluster analysis or automatic classification is the name given to a range of techniques for the grouping of multidimensional datasets. Although differing in their individual details, these methods are based on the grouping together, or clustering, of the most similar objects or pairs of objects. The similarities are typically calculated using nearest-neighbor searching routines (92). Various similarity searching strategies have been applied to identify an adequate subset of large databases for biological testing while retaining the assurance that compounds with novel shapes or properties have not been overlooked.

A shape-based clustering method has recently been shown to be useful for reducing the size of a database while retaining the geometrical diversity (42). When applied to test databases, timing studies indicate that the method is applicable to large datasets. One advantage of the distance method is that, for the same compound, conformational differences will be evident. Although this method does not require precomputing the conformations and exacerbates the problem of already large overhead of data storage. The connectivity method clusters together molecules of similar connectivity, without dependence on the conformation of the molecules, and therefore may not be an adequate representation of shape in systems capable of extending over a wide range of distances with a high degree of flexibility.

The application of the shape similarity approach, using atom triplets as descriptors, has also been used for rapid quantitative shape matching between two molecules or molecules and a template (93). The overall similarity is measured as a scoring factor between atom triplets.

Conclusions - There has been considerable progress in access and retrieval of 3D structural database information to aid in finding and designing new biologically active compounds. Three-dimensional database retrieval methods can already take into account the conformational flexibility of molecules. Efficient similarity searching methods are now aiming toward direct application to structure-activity and molecular design, wether at the level of small organic molecules or protein structures. In the current docking methods limited conformational flexibility, when addressed, is restricted to the ligand molecules. In general, docking or de novo strategies depend on the accuracy of the binding site. While the success of many of the methods depends on the starting

localization of the ligand with respect to the binding site, efficient sampling of the binding protein within reasonable computational limits needs improvement. Future developments will no doubt address these limitations.

STRUCTURAL DATABASES AND SIMILARITY SEARCHES

PROGRAMS	AFFILIATION	Reference
MOLPAT	Merck	10
QUEST3D/VISTA	Cambridge Crystallographic Database Centre	13
MACCS3D	Molecular Design Limited	13,14
CHEMDBS3D	Chemical Design Ltd.	15
UNITY	Tripos Associates, Inc.	16
CAST	Chemical Abstracts Service	17
FCD	Fine Chemical Directory	6
CONCORD	Tripos Associates, Inc.	18,19
COBRA/WIZARD	NA	20
ALADDIN	Daylight Chemical Information Systems	21
EMBED	NA	22
ASP	Oxford Molecular Limited	28-31

STRATEGIES IN 3D SIMILARITY SEARCHING

PROGRAMS	APPLICATION - VALIDATION	Reference
ASP	steroids	32
MEPCONF	monoamine oxidase B substrates	33
SPERM	dihydrofolate reductase	38
	DNA binding molecules	39
SUPER	leukotriene D4 receptor antagonists	45
RECEPS/AUTOFIT	dihydrofolate reductase ligands	46
YAK	carbonic anhydrase, thermolysin	47
DISCO	dopaminergic and benzodiazepine agonists	48
KEY/LOCK/LOCKSMITH	hemoglobin allosteric modifiers	49
CAVITY	HIV-1 protease	50
POCKET	adipocyte lipid binding protein, malate dehydrogenase	51
GEPOL	alcohol dehydrogenase, ribosomal protein	53
DOCK	papain, carbonic anhydrase	58
	alpha-chymotrypsin	59
DOCK2	trypsin, chymotrypsin, subtilisin	60
	thymidylate synthase	61
YING	dihydrofolate reductase, p-hydroxybenzoate hydroxylate, catabolite gene activator protein, phosphoglycerate kinase, ribonuclease T1	65
DISDOCK	serine protease complexes	66
AUTODOCK	immunoglobulin McPC 603, chymotrypsin, lysozyme, aconitase	67
CAVEAT	tendamistat, somatostatin analogs	75
FOUNDATION	thermolysin inhibitor	76
GROW	aspartyl proteases	77
LUDI	HIV protease, dihydrofolate reductase	78
DELEGATE(BUILDER)	HIV-1 protease	79
CLIX	L226Q influenza-virus hemagglutinin mutant	80
GRID	phospholipase-A2, dihydrofolate reductase, insulin	81
GenStar	FK-506 binding protein, HIV protease, carbonic anhydrase	82
LEGEND/LORE	dihydrofolate reductase	83
PROTEP	ubiquitin, thioredoxin, heat shock protein	91

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Chapter 30. Peptide Stability in Drug Development: *In vitro* Peptide Degradation in Plasma and Serum

Michael F. Powell
Pharmaceutical Research and Development
Genentech, Inc., South San Francisco, CA 94080

Introduction - The pharmaceutical development of peptides presents unique hurdles not found for either small molecule or protein therapeutics. Many of these impediments are directly related to peptide stability, or rather the chemical, physical, and *in vivo* enzymatic instability of peptides. This chapter briefly covers chemical and physical stability concerns reviewed recently including: general stability of pharmaceuticals (1,2); the effect of formulation stabilizers on peptide stability (3); protein/peptide degradation pathways (4); analytical methods to study polypeptide degradation (5); and peptide adsorption (6). Probably the greatest problem in using peptides as drugs is the nature of peptides themselves since there exists a whole class of molecules bent on peptide destruction - the peptidases. Even if efficient peptide delivery is achieved, many peptides do not circulate in blood for more than a few minutes due to enzymatic degradation, thus preventing their usefulness as therapeutic agents. It is critical in peptide drug development to determine which peptide candidates are enzymatically stable. The focus of this review is methodology for measuring peptide biostability - the *in vitro* degradation of peptides in serum and plasma. To date, there are no reviews of peptide stability in serum or plasma, and so a comprehensive summary of *in vitro* degradation half-lives upon which this method is based and understood is included.

CHEMICAL AND PHYSICAL PEPTIDE STABILITY

Chemical stability - In general, most peptide drugs are comprised of fairly stable building blocks - the amino acids, which might lead to the naive conclusion that chemical instability is not an issue. However, there are several chemical reactions which may occur for the 'average' peptide: hydrolysis, deamidation, isomerization, diketopiperazine formation, oxidation, photodecomposition and disulfide exchange (3). The combined effect of these reactions can be devastating. For example, if after two years, each pathway contributed only 1.5% towards peptide degradation in a parenteral formulation, the combined loss would be 10.5%, resulting in a shelf-life of less than two years. Also, there are many available sites for the identical reaction. A moderately sized peptide such as luteinizing hormone-releasing hormone (LH-RH, pyro-EHWSYGLRPG-NH_2) has nine backbone amide bonds, and all are subject to hydrolysis (albeit at different rates). LH-RH also has three photoreactive amino acids (histidine, tryptophan and tyrosine), thus producing a statistical minimum of six photoproducts. Many of these degradation pathways and their reaction kinetics are well understood, and have resulted in a few general rules. Peptide amide bonds formed by the amino group of serine or threonine have been found to be more labile than many other amide bonds (7,8). Deamidation of asparagine is usually faster than for glutamine, and is fastest when asparagine or glutamine is bonded to glycine(9). Racemization is fastest for asparagine, tyrosine, serine and phenylalanine because the electron-withdrawing side groups stabilize the carbanion intermediate in this reaction (10). Oxidation of histidine, tyrosine, cysteine and tryptophan is generally faster at higher pH's, whereas methionine oxidation often shows less pH dependence. The observed reaction rate is often governed by trace amounts of catalysts including peroxide, metal ions, light, base and free radical initiators (11). These and other observations can help pharmaceutical scientists design optimal formulations for peptide stability and delivery.

Physical Stability - There are two types of physical instability that peptides occasionally exhibit: adsorption to surfaces and peptide aggregation. Surface adsorption of peptides is a problem at low concentration, especially for hydrophobic peptides such as the LH-RH analog nafarelin (12). Adsorption is a case where peptide behavior resembles protein behavior; many proteins in aqueous solution show adsorptive losses at concentrations below 10-100 µg/mL (13). Peptide fragments of secretin have shown binding to plastic surfaces including catheters (14). Prevention of peptide adsorption can often be prevented by increasing peptide concentration. Overcoming peptide aggregation, however, has not been quite as simple. Hydrophobic peptides in concentrated

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aqueous solution tend to aggregate, forming liquid crystals, gels and precipitates. The nature of peptide aggregation has been studied in detail for pentagastrin (15), and for a few LH-RH analogs(16). The parent peptide LH-RH did not aggregate to form liquid crystals, whereas some of the more hydrophobic analogs formed liquid crystals after a few days (16). Peptide aggregation was suppressed by lowering the peptide concentration, by adding organic cosolvents, or by using hydrophilic salts (such as acetate) as buffering agent, instead of chloride or phosphate (17). Adding cyclodextrins also decreased peptide aggregation, presumably by sequestering the hydrophobic side chains (18). Physical instability of peptides has not received much attention, but will become more important as more peptides are put into the pharmaceutical pipeline.

PEPTIDE STABILITY IN PLASMA AND SERUM

Rationale - The prediction of *in vivo* peptide stability has been quite difficult because several factors are involved: absolute absorption or permeability, first pass metabolism, hepatic and renal clearance, cellular binding and uptake, and stability towards circulating peptidases. Some of these, such as renal or hepatic clearance, may be estimated accurately only by actual measurement of *in vivo* clearance rates. Since animal experiments are expensive, labor-intensive, and often show great variability, several *in vitro* models for determining peptide stability have been developed. For example, *in vitro* permeability measurements through epithelial gut tissue are very useful for determining if a peptide is likely to be orally absorbed (19). In order to estimate *in vivo* peptide clearance by circulating peptidases, several researchers have measured *in vitro* peptide stability in plasma or serum. This measurement has become one of the more important pharmaceutic assays in peptide drug development because it identifies one of the contributing factors to short biological half-life, and shows which peptides are likely to be therapeutically ineffective (20). These measurements are usually carried out by adding a small amount (1-100 μM) of peptide to serum or plasma at 37 °C, then removing aliquots at known time intervals and assaying for remaining peptide by HPLC or immunoassay. In contrast to proteins, these two methods are generally sufficient because they are sensitive to modifications in primary chemical structure.

Limitations - Although the kinetic determination of peptide stability is straightforward, several experimental factors have been found to produce misleading results. The use of non-human serum or plasma often gives different results than human serum. Levels of certain peptidases (such as prolyl-endopeptidases (21)) vary across species resulting in disparate *in vitro* peptide stabilities (22,23). For example, thyrotropin releasing hormone (TRH) pyro-EHP-NH_2 degrades more than 100-fold faster in pig plasma than in dog plasma (24), and the oxytocin derivative PLG-NH_2 reacts 200-fold faster in rat plasma than in human plasma (25). In general, there appears to be little correlation with reactivity and phylogenetics, at least for the peptides studied to date. One generalization has been observed, however, in that peptide degradation is typically faster in rat plasma than in human plasma or serum, as shown in Figure 1. Peptide stability measurements in animal plasma or serum are very useful for interpreting animal study data, but they should be used with extreme caution to predict peptide stability in human plasma.

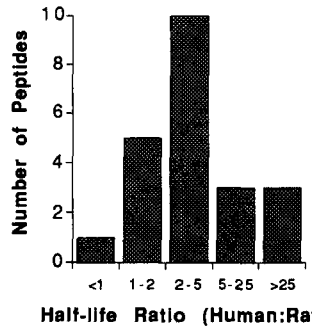


Figure 1. Histogram showing relative peptide reactivity in human plasma and rat plasma (or serum). (Data obtained from Table 1). Most peptides are more reactive in rat than human plasma.

Inter-subject variation of plasma or serum is also important. For example, thyrotrophin in human serum showed a half-life of 5 to 37 minutes when using sera from individual subjects (26). The half-life also varied with age of the subject where the degradation rate was approximately twice as fast in children as for adults (27). Certain disease states, such as pancreatitis (28), also affect circulating peptidase levels, as well as levels of other enzymes including amylase, isoamylase, lipase and trypsin (29). Adult respiratory distress syndrome causes a 50-fold increase in neutral endopeptidase serum levels (30). Aminopeptidase levels are also dependent on disease state (31).

In order to prevent coagulation, EDTA, citrate, or heparin are added to plasma. Peptide degradation rates may be artificially slow when measured in plasma with added EDTA because EDTA acts as a peptidase inhibitor for metal ion-dependent peptidases. For example, LH-RH degraded with a half-life of 9.6 h in heparinized plasma, but showed a half-life of 47 h in plasma

prepared with EDTA (23). Adding EDTA also inhibited the *in vitro* degradation of both CCK-33 and CCK-8 (32). Degradation of delta sleep-inducing peptide (DSIP) was also strongly inhibited by the addition of EDTA (33). TRH degradation in human or rat serum was inhibited by 1-10 mM EDTA (34). Indeed, EDTA has been used to prepare samples for radioimmunoassay to retard degradation, e.g., atrial natriuretic peptide (ANP) (35).

A stability-specific assay method (one that is able to differentiate between the substrate and its degradation products) is also required to determine peptide stability. Lack of stability-specificity gave different peptide half-lives for the immunoassay (63 minutes) and HPLC (17 minutes) detection of growth hormone-releasing hormone (36). TRH in human serum exhibited a half-life of 17 minutes by radioimmunoassay (26), and 1300 minutes by monitoring the rate of histidine formation (37). This is not surprising, considering the primary degradation pathway of TRH in plasma (cleavage of the pyro-Glu--His bond) does not form histidine (38). The long half-life reported for PLG-NH_2 (25) also suffered from this drawback because the major degradation pathway of PLG-NH_2 in human serum did not afford the monitored product, G-NH_2. Artificially-long half-lives may also be detected by using a bioassay for determining *in vitro* half-lives, as has been done for RGD-containing peptides (39), since the peptide may degrade to give a product which is still bioactive. On the other hand, loss of the radiotracer by a label-specific pathway results in half-lives which are too short (40). Enzyme saturation may also occur in experiments of this type if the peptide concentration is high, typically more than 100 μM. Pseudo first-order kinetics were observed for TRH concentrations up to 100 μM, where the rate was independent of peptide concentration and linearly proportional to plasma or serum concentration (41,42). This has also been verified by comparison of radiolabeled and cold TRH degradation rate constants in serum. TRH exhibited good pseudo first-order kinetics at 10 μM substrate with a half-life of 10 minutes; radiolabeled TRH at 10 nM showed similar kinetics and half-life (17 minutes) (26). At higher peptide concentrations, for example 500 μM TRH, reaction kinetics were no longer pseudo first-order, indicating possible enzyme saturation (38).

General Observations - The peptide stability data in Table 1 consist of peptide reactivity in serum and plasma recorded to date, and are the basis for a number of observations. First, peptide reactivity in heparinized plasma and serum is quite similar, where the mean ratio of serum:plasma half-life is 1.08 ± 0.22 (this includes *all* peptides studied in both media). Further evidence for similar peptide reactivities is provided by comparison of aminopeptidase (AP) activity, as measured by proteolysis of substituted β-naphthylamides in plasma (prepared with ammonium oxalate) (43) or serum (44). Figure 2 shows that the arylaminopeptidase activity of plasma and serum is proportional (as referenced to Leu-β-naphthylamide), at least for AP's that cleave leucine, methionine, phenylalanine, arginine, lysine and alanine. These results are important because most of the peptidase activity has been reported to be AP-like in nature (*vide infra*). Based on these similar AP activities, and the similar reactivity of more than 20 different peptides in plasma and serum, it is generally accepted that either serum or heparinized plasma may be used for estimating the stability of peptides towards circulating peptidases. Notably, few stability studies have been carried out in whole blood, presumably because of the difficulty in peptide isolation. In the few cases reported, for example, CCK-58 (28), TRH (45), and enkephalin (46), the degradation rates in blood are fairly similar to those observed in plasma or serum. Many of the peptides show extremely short half-lives, some only a few minutes, which preclude their therapeutic usefulness. Most peptides have half-lives of 15 minutes or less. There are a few cases where long peptide half-lives were observed and are attributable to stabilization by chemical modification or the method of analysis used, such as detection of free amino acids. In general, most replicate analyses of the same peptide, even by different researchers, gave similar results.

Peptide modification (with amide bond isosteres or D-amino acids) often increases peptide stability significantly, especially if more than one modification is made. A particularly notable case of non-additive stabilization has been reported for the *in vitro* half-lives of thymopentin analogs: RPDVY, 1 min; RPDVY-NH_2, 1 min; Ac-RPDVY, 40 min; and Ac-RPDVY-NH_2, no detectable degradation (47). Another example is the dipeptidylaminopeptidase (DPAP) cleavage of GRF in plasma. Modification of GRF(1-29)-NH_2 with Ala^{15} did not increase the 15 minute half-life as expected, but substitution with cyclo-Lys^{21}-Asp^{25} (KLLQD) increased the half-life to >240 minutes (48).

YADAIFTNSYRKVLAQLSARKLLQDIMSR-NH_2 → YA + DAIFTNSYRKVLAQLSARKLLQDIMSR-NH_2

This is somewhat unexpected because the cyclo-Lys^{21}-Asp^{25} modification is quite distant from the site of cleavage. Another interesting example of long-range stabilization has been reported for Leu-enkephalin (49). Isosteric amide bond replacement, even when the CH_2S isostere is distally removed from Tyr-Gly primary cleavage site, affords significant stabilization (50,51).

Table 1. Summary of Peptide Half-lives in Serum, Plasma or Blood at 37 °C.

Peptide Analog	Sequence[a]	Media[b]	Species[c]	t1/2[d]	Notes	Ref
Atrial Natriuretic Factor	*ANF*(99-126)	B,P	R	>60		52
	ANF(1-126)	S	R	<10		53
Cholecystokinin	WMDF-NH2	P	R	<1		54
Analogs	WMDF-NH2	P	H	13		"
	YMGWMDF-NH2	P	R	2.5		"
	YMGWMDF-NH2	P	H	~5		"
	DYMGWMDF-NH2	P	R	5		"
	DYMGWMDF-NH2	P	H	18		"
	DY(SO3H)MGWMDF-NH2	P	R	17		"
	DY(SO3H)MGWMDF-NH2	P	H	50		"
	RDY(SO3H)YGWLDF-NH2	P	H	2.5		"
	DRDY(SO3H)MGWMDF-NH2	P	R	45		"
	DRDY(SO3H)MGWMDF-NH2	P	H	30		"
	CCK-58	B	H	70		28
	CCK-58	P	H	45		"
	CCK-58	B	H	11	e	"
	CCK-58	P	H	10	e	"
Dermorphin Analog	YaFGYPS-NH2	P	H	>180		55
	YaFGYPS-NH2	P	R	>180		"
DSIP Peptides	WAGGDASGE	S,P	R	~2	f,g	33
	WAGGDASGE	S,P	H	~5-10	f,g	"
Enkephalin	YGGFL	S	H	7		49
Analogs	Y[CH2S]GGFL	S	H	47	f	"
	YG[CH2S]GFL	S	H	35	f	"
	YGG[CH2S]FL	S	H	67	f	"
	YGGF[CH2S]L	S	H	157	f	"
	YaG[CH2S]FL	S	H	>500	f	"
	YaGFL	S	H	63	f	"
	YGGFL	P	R	0.5		51
	YGGFM	P	R	0.4		"
	YGGFL	P	H	1.3	f	56
	Y(SO3H)GGFL	P	H	ndo		57
	YGGFL	P	H	15		50
	YGGFL	P	H	5		55
	YGGFL	P	R	2		"
	YGGFL	S	O	4	g,h	58
	YGGFL	P	H	7	g,h	59
	YGGFL	P	M	10		"
	YGGFL	P	R	0.8		"
	YGGFL	P	C	0.3		"
	YGGFL	P	O	1.9		"
	YGGFL	P	L	1.5		"
	YGGFL	B	R	1.7		46
	YGGFL	P	R	1.6		"
	GGFL	B	R	9		"
	GGFL	P	R	5		"
	YGGFL	P	L	1.5		60
	YGGFL	P	M	9		61
	YGGFM	P	M	5		"
Endorphin DEE	TSEKSQTPLVTL	P	H	19	g	62
	TSEKSQTPLVTL	P	R	16		"
	TSEKSQTPLVTL	P	D	13		"
FGF Peptide	PALPEDFFSY	S	H	5.5	f,g,i	63
Gastrin releasing Peptide	Ac-HWAVGH[Emp]	S	H	ndo		64
Glutamine Dipeptides	GQ	P	H	46		65
	AQ	P	H	550		"
Growth Hormone-	*Leu*27-*GRF*-NH2	P	P	8		66
Releasing Factor	*Leu*27-*GRF*-NH2	P	B	22		"
	GRF-NH2	P	P	13		48
	Ala15-GRF-NH2	P	P	17		"
	des-NH2-*Tyr1,Ala15-GRF*-NH2	P	P	60		"
	des-NH2-*Tyr1,ala2,Ala15-GRF*-NH2	P	P	60		"
Growth Hormone-	*GRH*(1-44)-NH2	P	H	(63)	h	36
Releasing Hormone	*GRH*(1-44)-NH2	P	H	17		"
	GRH(3-44)-NH2	P	H	55		"
	GRH(1-40)	P	H	24		"

Table 1 Continued. Summary of Peptide Half-lives in Serum, Plasma or Blood at 37 °C.

Peptide Analog	Sequence[a]	Media[b]	Species[c]	t1/2[d]	Notes	Ref
LH-RH Analogs	<EHWSYGLRPG-NH2	S	H	(435)	f,h	37
	<EHWSYGLRPG-NH2	S	R	(90)	f,h	"
	<EHWSYGLRP-NHEt	S	H	(160)	f,h	"
	<EHWSYGLRP-NHEt	S	R	(240)	f,h	"
	<EHWSYaLRP-NHEt	S	H	(6,400)	f,h	"
	<EHWSYaLRP-NHEt	S	R	(3,100)	f,h	"
	<EHWSYGLRPG-NH2	S	B	720		23
	<EHWSYGLRPG-NH2	S	H	500		"
	<EHWSYGLRPG-NH2	S	R	350		"
	<EHWSYGLRPG-NH2	S	C	210		"
	<EHWSYGLRPG-NH2	P	B	415		"
	<EHWSYGLRPG-NH2	P	H	575		"
	<EHWSYGLRPG-NH2	P	R	440		"
	<EHWSYGLRPG-NH2	P	C	160		"
MHC Binding Peptides	HIATNAVLFFGR	S	H	4	f,g	41
	YLEDARRLKAIYEKKK	S	H	3	f,g	"
	NGQIGNDPNRDILY	S	H	32	f,g	"
	VITAFSDGLK	S	H	2	f,g	"
	QKQEPIDKELYPLTSL	S	H	12	f,g	"
	CPKVYRSAKLRM	S	H	3	f,g	"
	RTLYQNVGTYVSVGTSTLNK	S	H	114	f,g	"
	DNVLDHLTGRSC	S	H	913	f,g	"
	IVWASRELERFAVNPG	S	H	4	f,g	"
	GRTQDENPVVHFFKNIVTPRTPPP	S	H	99	f,	"
	QYIKKNSKFIGITE	S	H	17	f,	"
	QYIKANSKEIGITE	S	H	7	f,g	"
	ARRLKAIYARRLK	S	H	1	f,g	"
	FNNFTVSFWLRVPKVSASHLEQY	S	H	47	f,g	"
	YTLLQAAPALDKLKLTGDEATGANI	S	H	948	f,g	"
	RILAGALATYQ	S	H	3	f,g	"
	HPPHIEIQMLKNGKKI	S	H	74	f,g	"
	TLLQAAPALDKY	S	H	9	f,g	"
	pyro-QYIKKNSKFIGITE	S	H	224	f,g	"
	QYIKANSKFIGKTE	S	H	21	f,g	"
	YPKFVKQNTLKLAT	S	H	28	f,g	"
	QYIKANSKFIGIFE	S	H	22	f,g	"
	TYIKANSKFIGITE	S	H	37	f,g	"
	QYIKANQKFIGITE	S	H	13	f,g	"
	QEIKANSKFIGITE	S	H	25	f,g	"
	QYIKANSKFKGITE	S	H	8	f,g	"
	QYIKANSKFIGITE	S	H	9	f,g	"
	PKYVKQNTLKLAT	S	H	27	f,g	"
	pKYVKQNTLKLAt	S	H	>180	f,g	"
MSH	*des*-Ac-*MSH*	P	O	28	j	67
	Ac-*MSH*	P	O	92		"
	di-Ac-*MSH*	P	O	416		"
Oxytocin	CYLQNCPLG-NH2	S	H	ndo		68
Analogs	PLG-NH2	S	H-f	6900	f	25
	PLG-NH2	S	H-m	2900		"
	PLG-NH2	S	R	21		"
	PLG-NH2	S	L	1800		"
	LG-NH2	S	H	61	f,h	"
	LG-NH2	S	R	14	f,h	"
	PL	S	H	77	f,h	"
	PL	S	R	<10	f,h	"
	PLG-NH2	P	H	8000		69
	PLG-NH2	P	R	26		"
RGD peptides	RGDS	P	H	15		70
	RGDY(Me)-NH2	P	H	20		"
	Ac-RGDY(Me)-NH2	P	H	ndo		"
	des-NH2-RGDY(Me)-NH2	P	H	ndo		"
	RGDS	P	H	17	h	71
	RGDY(Me)-NH2	P	H	20	h	"
	RGDY(Me)-NH2	P	R	20	h	"
	RGDY(Me)-NH2	P	D	11	h	"
	Ac-RGDY(Me)-NH2	P	H	>200	h	"

Table 1 Continued. Summary of Peptide Half-lives in Serum, Plasma or Blood at 37 °C.

Peptide Analog	Sequence[a]	Media[b]	Species[c]	t1/2[d]	Notes	Ref
	des-NH2-RGDY(Me)-NH2	P	H	>200	h	"
Somatostatin	AGCKNFFWKTFTSC	P	H	3	h	72
Analogs	cyclo-NFw[Gaba]	B	R	ndo		73
	Dihydro-somatostatin	S	H	(200)	f,h	37
	Dihydro-somatostatin	S	R	(75)	f,h	"
	Ac-GCKNFFWKTFTSC	S	H	(440)	f,h	"
	AGCKNFFwKTFTSC	S	R	(1300)	f,h	"
Substance P	RPKPQQFFGLM-NH2	P	H	(70)	k	74
Thymopentin	RKDVT	S	H	~1		47
Analogs	RPDVT	S	H	~1		"
	RPDVT-NH2	S	H	~1		"
	Ac-RPDVT	S	H	~35		"
	RkDVT	S	H	~40		"
	Ac-RKDVT-NH2	S	H	~45		"
	R[Aib]DVT	S	H	~30		"
	R[Aib]DVT-NH2	S	H	ndo		"
	Ac-RPDVT-NH2	S	H	ndo		"
	RKDVT	P	H	0.5	g	75
Thyrotropin Releasing	<EHP-NH2	S	H	(1300)	f,h	37
Hormone Analogs	<EHP-NH2	S	R	(230)	f,h	"
	<E(3-Me)HP-NH2	S	H	(40)	f,h	"
	<E(3-Me)HP-NH2	S	R	(30)	f,h	"
	HP-NH2	S	R	(255)	f,h	"
	<EHP-NH2	P	M	114	f,g	24
	<EHP-NH2	P	K	85		"
	<EHP-NH2	P	R	13		"
	<EHP-NH2	P	C	180		"
	<EHP-NH2	P	O	62		"
	<EHP-NH2	P	F	11		"
	<EHP-NH2	P	D	>1500		"
	<EHP-NH2	P	S	47		"
	<EHP-NH2	P	G	74		"
	<EHP-NH2	P	H	18		"
	<EHP-NH2	P	P	4		"
	<EH[Dmp]-NH2	P	H	540	f	76
	<EH[Dmp]-NH2	P	R	195		"
	<EH[Dmp]-NH2	P	D	>1500		"
	<EH[Dmp]-NH2	P	M	>1500		"
	<EHP-NH2	P	H	10		38
	<EHP	P	H	27		"
	<EHP-NH2	S	H	17	g,j	26
	<EHP-NH2	S	H	15	j	77
	<EH(3-Me)P-NH2	S	H	34	j	"
T Peptides	ASTTTNYT	P	R	5.2		78
	STTTNYT	P	R	3.5		"
	TTTNYT	P	R	2		"
	TTNYT	P	R	1.1		"
	TNYT	P	R	1		"
	NVT	P	R	1		"
	VT	P	R	1		"
	ASTTTnYT	P	R	5.3		"
	ASTTTNY[Aib]	P	R	5.3		"
	AST*T*[Aib]NYT	P	R	5.2		"
	AST[Aib]TNYT	P	R	5.1		"
Vasopressin Analogs	CYFQNCPRG-NH2	S	R	920	f,h	79
	CYFQNC[Sar]RG-NH2	S	R	135	f,m	"
	CYFQNCPR	P	H	>720	g	22
	CYFQNCPR	P	R	350		"
	CYFQNCPR	P	D	100		"
	[Mpa]YFQNCPrG-NH2	P	H	>2000		80

a) The following descriptors are used: L- amino acids are represented using the standard single letter codes in upper case; D-amino acids are in lower case; peptides terminated with 'Ac-' or '-NH2' represent the N-terminal acetylated or C-terminal amide derivatives, respectively; '-NHEt' stands for the C-terminal N-ethyl amide; square brackets indicate the use of an amide bond isostere or amino acid replacement; *parentheses indicate a*

Table 1 Continued. Summary of Peptide Half-lives in Serum, Plasma or Blood at 37 °C.

substitution on the amino acid preceding the parentheses; '<E' represents pyro-glutamic acid; sequences in italics (followed by numbers only in parentheses) represent the peptide by its general name and peptide number (ala denotes D-alanine); 'Aib' stands for aminoisobutyric acid; 'Gaba" stands for gamma aminobutyric acid; 'Emp' = (S)-1-ethoxy-4-methyl-2-pentane; 'Dmp' = 3,3-Dimethylproline; 'Sar' = sarcosine; 'Mpa' = 3-mercaptopropanoic acid. A solid underline indicates cyclization via the last amino acids indicated, where cysteine-cysteine cyclization is via disulfide formation, and lysine-glutamic acid is via lactam formation. Common peptide names (where the sequence is too long to show) are given in italics. **b**) 'S' = serum; 'P' = plasma (heparinized); 'B' = blood **c**) 'H' = human ('f' = female, 'm' = male); 'R' = rat; 'D' = dog; 'M' = mouse; 'B' = bovine; 'K' = hamster; 'F'= feline; 'S' = monkey; 'G' = goat; 'C' = guinea pig; 'P' = pig, 'O' = rabbit, 'L' = chicken **d**) Half-lives are given in minutes. The half-lives in parentheses represent the maximum value of half-life because only a single amino acid appearance was monitored; the true half-life may actually be much shorter. These data should be used only for intra-comparison purposes only. Where only approximate values were determined the half-life values are preceded with a '~'. Where no degradation was observed, 'ndo' is used. **e**) From patients with acute pancreatitis **f**) Corrected for dilution factor with buffer **g**) Radiolabeled peptide **h**) Calculated from raw data in the text **i**) Carried out at 4°C - not corrected to 37° C **j**) Immunoassay **k**) Bioassay **m**) Other 1- and 7-substituted positions were also studied (data not shown).

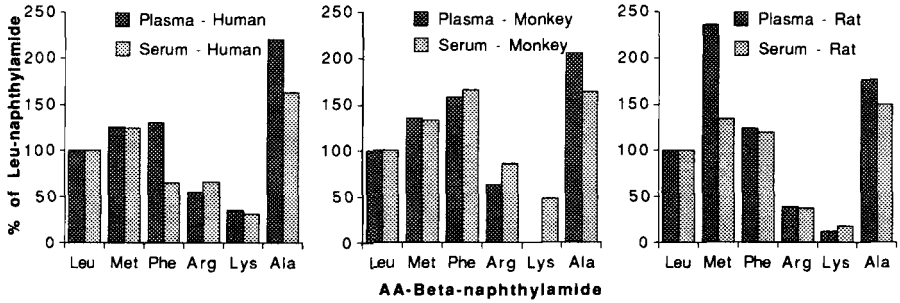


Figure 2. Comparison of Human, Monkey and Rat Aminopeptidase Activity in Serum and Plasma at 37 °C (Reference = Leu-β-naphthylamide; monkey plasma results for Lys not reported) (43,44)

Mechanisms of Peptide Degradation in Serum and Plasma - Inspection of the data in Table 1, as well as from peptidase inhibitor studies included therein, show that most L-amino acid peptides that are not N-terminally modified degrade by AP or DPAP activity. Enkephalin degraded in human plasma by AP cleavage to give free tyrosine (YGGFL → Y + GGFL) (50). This reaction was inhibited by AP inhibitors such as bestatin or puromycin (56). Analogs such as Y(SO_3H)GGFL (57) and YaGFL (49) were much more stable than the parent peptide. Somatostatin was cleaved by AP's, where N-terminally modified analogs were more stable (72).

AGCKNFFWKTFTSC → A + GCKNFFWKTFTSC

Similarly, the IIbIIIa antagonists RGDY(Me) degraded by AP activity to give R and GDY(Me). Modification of RGDY(Me) by N-terminal acetylation or use of AP inhibitor bestatin prevented degradation (70,71). Degradation of thymopentin RKDVY occurred at the R-K bond, and N-terminal acetylation, or D-lysine, proline or aminobutyric acid substitution for K resulted in significantly longer half-lives (47). DSIP degraded in serum or plasma by AP activity, and showed a good correlation with Leu-AP activity (33). Conversion of glutamic acid to pyroglutamic acid increased the half-life of tetanus toxin 830-843 peptide in human serum from 17 minutes to 224 minutes, indicative of AP or DPAP cleavage (41). TRH degraded by AP cleavage, however in this case a specific pyroglutamyl-AP was found to be the active agent (81). GRH(1-44)-NH_2 degraded primarily via DPAP activity to give the GRH(3-44)-NH2 variant (YADAIFTNS... → YA + DAIFTNS...). The TP5 peptide cleaves in human plasma by both AP and DPAP activity (47). Several of the cholecystokinin peptides have been reported to degrade by AP activity, as evidenced by product isolation and enzyme inhibitor studies (54).

DY(SO_3H)MGWMDF-NH_2 → D + Y(SO_3H)MGWMDF-NH_2

The T peptides (see Table 1) also cleaved primarily by AP activity as determined by AP inhibitor and product identification studies (78). It should be kept in mind that serum or plasma AP is really a

mixture of AP's; for example, Leu-AP cleaves N-terminal Leu from different substrates at different rates (82). These data suggest that the primary degradation route of many small peptides in serum or plasma is by exopeptidase activity, mostly by AP or DPAP activity. This is not a hard and fast rule, even for the same peptide. The mechanism of proteolytic cleavage may be species-dependent, as observed for enkephalin degradation in human plasma (by AP's) or rabbit plasma (by DPAP's and dipeptidylcarboxypeptidases) (59). DEE also reacts by different pathways; in dog serum or plasma the predominant pathway is via AP activity, whereas in rats and humans the carboxypeptidase (CP) pathway predominates (62).

Evidence for predominant endopeptidase cleavage was found for only specific polypeptides (such as ANF where cleavage afforded an active compound), or where the N- and C-termini were modified (52,53). LH-RH is protected at the termini and thus reacts by endopeptidase cleavage, primarily at two sites (23).

$$\begin{aligned} \text{<EHWSYGLRPG-NH}_2 &\rightarrow \text{<EHW} + \text{SYGLRPG-NH}_2 \\ &\rightarrow \text{<EHWSY} + \text{GLRPG-NH}_2 \end{aligned}$$

The D-alanine analog of dermorphin is also cleaved by endopeptidase activity, presumably because AP (or in this case, DPAP activity, because of the N- terminal YA amino acids) activity is eliminated by the D-amino acid substitution, and CP activity is reduced by the C-terminal amide (55).

$$\text{YaFGYPS-NH}_2 \rightarrow \text{YaFG} + \text{YPS-NH}_2$$

Many other N- and C-termini protected peptides, including gastrin-releasing peptide (Ac-HWAVGH[Emp]), oxytocin, Ac-RGDY(Me)-NH_2, des-NH_2-RGDY(Me)-NH_2, R[Aib]DVT-NH_2, and <EH[Dmp]-NH_2 were found to be completely resistant to peptidase degradation, even though these peptides were not designed specifically to block endopeptidase cleavage.

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Chapter 31. Adenosine Receptors as Pharmacological Targets

Mark D. Erion
Gensia Pharmaceuticals, Inc., San Diego, CA 92121

Introduction - Adenosine receptors are expressed on the surface of nearly all cells (1). Activation of adenosine receptors produces cell-specific physiological responses that are potentially useful for the treatment of a variety of diseases, including hypertension, myocardial and cerebral ischemia, arrhythmia, thrombosis, seizure, pain, anxiety, neurodegeneration, diabetes and inflammation (2). Despite the therapeutic potential of adenosinergic compounds, the only drugs currently marketed are adenosine itself for the treatment of supraventricular tachycardia and the adenosine transport blocker dipyridamole as an adjunct to warfarin for the prevention of postoperative thromboembolic events after heart valve surgery. The overall lack of success to date stems from the ubiquitous nature of adenosine receptors, the profound pharmacology evoked upon receptor activation and the absence of compounds that only activate adenosine receptors expressed on the target tissue (3). Discovery of adenosinergic compounds with greater pharmacological specificity is anticipated, however, from recent studies demonstrating the existence of several distinct adenosine receptor subtypes (4) and from studies aimed at finding compounds that increase endogenous adenosine concentrations to pharmacologically active levels only at a specific site (*e.g.*, ischemic regions in the heart) and only after a specific event (*e.g.*, ischemia) (5). This chapter will review these studies as well as the recent literature on adenosine receptor structure-function and the pharmacology associated with adenosinergic compounds.

Receptor Structure-Function - Adenosine receptors are part of the G-protein-coupled receptor superfamily based on molecular cloning and sequencing studies (6). Sequence homology to other G-protein coupled receptors indicates that adenosine receptors have the classical seven-transmembrane-helix topology, an extracellular amino terminus, a cytoplasmic carboxyl terminus containing phosphorylation sites and a cytoplasmic domain that could interact with the G-protein (7).

Five receptor subtypes have been identified, namely A1, A2a, A2b, A3 and A4, based on ligand-binding, signal transduction pathway and sequence homology criteria (4). The first adenosine receptors cloned were A1 and A2a. The clones were obtained by screening a canine thyroid cDNA library by PCR using degenerate oligomers directed at the transmembrane domains of G-protein coupled receptors (8,9). Identification of the A1 and A2a subtypes was accomplished by expressing the PCR transcripts in cells and showing that, in one case, A1 agonists bind and inhibit forskolin-stimulated cAMP production, whereas in the other case, A2 agonists bind and lead to the activation of adenylyl cyclase. Amino acid sequence identity between subtypes A1 and A2a is about 63% in the putative transmembrane regions (50% sequence identity overall) whereas it is less than 30% with other seven transmembrane receptors (10). The A1, A2a and A2b receptors have been cloned from other species, including rat (11-14), bovine (15,16) and human (17-19). Sequence homology is high across species (> 90%). The fact, however, that the bovine, rat and canine A1 receptors exhibit high homology but different SAR suggests that minor sequence differences can produce significant effects (4). Selective agonists and antagonists have been described that help differentiate the receptor subtypes. Comprehensive reviews covering selective A1 and A2 agonists and antagonists have been published in the last few years (20,21). Recently, a novel adenosine receptor, classified as the A3 receptor, was cloned, expressed and shown to bind A1-type agonists such as N^6-2-(4-amino-3-phenyl)ethyladenosine, but not A2 agonists or A1-selective xanthine-based antagonists such as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (22).

Activation of adenosine receptors produces a variety of biochemical and electrophysiological effects that in general lead to a decrease in cellular activity and cellular excitability (Table 1). Adenosine exerts its effect on the cell through receptor-mediated cell signaling pathways (23). The specific pathway involved is dependent on the receptor subtype and cell type. Originally, two receptor subtypes, namely A1 and A2, were identified and shown to inhibit and activate cAMP production via adenylyl cyclase, respectively (24). Adenylyl cyclase activity is modulated by adenosine receptor activation through G-regulatory proteins. More recently, other signaling pathways have been discovered that are independent of cAMP concentration and involve A1

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receptor associated G-proteins that interact with ion channels (25). For example, activation of A1 receptors located on presynaptic terminals in the hippocampus leads to a decrease in calcium influx through voltage-sensitive channels. Postsynaptic neurons, on the other hand, and the conduction system within the heart, increase potassium conductance after A1 receptor stimulation.

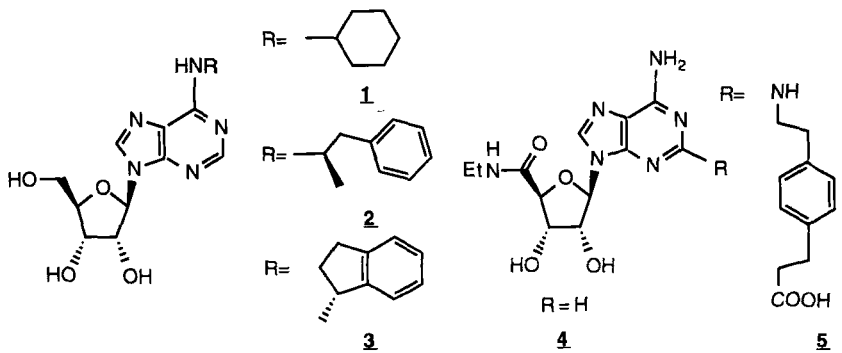
TABLE 1: ADENOSINE RECEPTOR FUNCTION[¥]

TISSUE	CELL	ADENOSINE ACTION	AR	SYS	REF
vascular	smooth muscle	vasodilation	A2a	AC	26
	endothelial	increases permeability	A2b?	AC	27
blood	platelet	antiaggregation	A2a	AC	28
	monocyte	inhibits SO generation	A2	AC	29
	lymphocyte	inhibits function, proliferation	A2		29
	basophil	inhibits histamine release	A2	AC	29
	neutrophil	inhibits adhesion	A2a		30
		inhibits SO generation	A2a		31
		promotes chemotaxis	A1		31
heart	AV nodal cell	prolongs impulse conduction	A1	K^+	32
	sinus nodal cell	decreases heart rate	A1	K^+	32
	atrial myocyte	decreases contractility	A1	K^+	32
brain	presynaptic neuron	inhibits glutamate release	A1	Ca^{++}	33
	postsynaptic neuron	inhibits hyperpolarization	A1	K^+	33
spinal cord	nociceptive neuron	antinociception	A2	AC	34
fat	adipocyte	inhibits lipolysis	A1	AC	35
kidney	smooth muscle	vasoconstriction	A1	Ca^{++}	36
	juxtaglomerular cell	inhibits renin release	A1	AC	36
GI tract	gastric cell	inhibits acid secretion	A1	AC	37

[¥]Abbreviations: AR, adenosine receptor subtype; Sys, signal transduction pathway; AC, adenylyl cyclase dependent; SO, superoxide; AV, atrioventricular

CARDIOVASCULAR PHARMACOLOGY

Hypertension - Adenosine and adenosine agonists produce a potent hypotensive response in hypertensive and normotensive animals (38). A1 agonists (*e.g.* CHA, **1**, or R-PIA, **2**) lower blood pressure by depressing cardiac conductance and contractility through activation of A1 receptors on nodal cells and cardiac myocytes (32). The blood pressure lowering effect of A1 agonists in



spontaneous hypertensive rats (SHR) is reported to correlate with the bradycardic response (39). A2 agonists, on the other hand, produce vasodilation of coronary arteries as well as the peripheral vasculature by activation of A2 receptors located on vascular smooth muscle cells (26). A potent selective A2 agonist, CGS 21680C (**5**) (40), was shown to decrease blood pressure in the SHR at 10 mg/kg p.o. for up to 24 h. without producing bradycardia (41). As in the case of other vasodilators, a transient tachycardia was induced by **5** presumably due to autonomic reflex mechanisms. Chronic treatment with **5** led to receptor down-regulation and loss of the hypotensive response (42). Other A2 agonists have been reported to have potent antihypertensive effects (43, 44). Some compounds that exhibit both A1 and A2 agonist properties (*e.g.*, **3**) have been reported to lower blood pressure without significantly increasing heart rate (39).

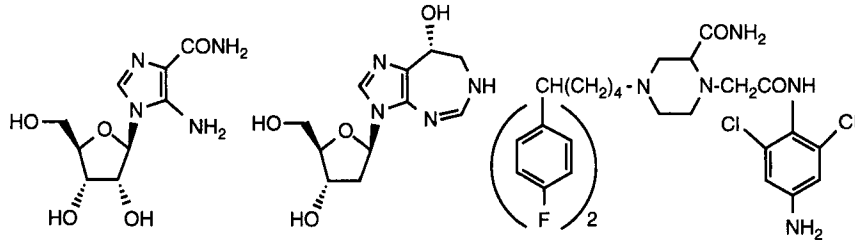
CAD Diagnosis - Regions of the myocardium with the potential for inadequate blood flow due to coronary artery disease (CAD) can be detected by two different methods. The classical method is to increase the oxygen demand by the heart and thereby induce a relative deficiency of blood flow to the diseased regions of the heart. These conditions are often met during strenuous exercise or during administration of a β-agonist such as arbutamine (45). Areas with insufficient blood flow become ischemic and exhibit dysfunction. The dysfunction is readily detected by ECG or echocardiography (detects wall motion abnormalities). The second method is to induce maximal vasodilation of coronary vessels so that perfusion differences in stenotic vessels are magnified and readily apparent by radionuclide imaging techniques. Both exercise and β-agonists lead to vasodilation and therefore can be used in combination with thallium-201 (or technetium-99 complexes) to detect CAD. Direct vasodilators such as adenosine and the adenosine transport inhibitor dipyridamole are also effective in combination with thallium-201 (46,47). In this case, the effect can occur in the absence of ischemia, although the diversion of blood flow away from stenotic arteries (known as "coronary steal") will often induce ischemia as evidenced by angina. Dipyridamole and adenosine produce vasodilation by an A2-receptor mediated relaxation of smooth muscle cells (26). A potential advantage for adenosine compared to dipyridamole is its rapid onset and offset of action (48). Administration of adenosine by continuous intravenous infusion is reported to produce maximal vasodilation without large effects on AV conduction, but with a moderate dose-dependent decrease in blood pressure and increase in heart rate (49). Data in CAD patients showed that thallium perfusion imaging was possible with adenosine and that consequently adenosine could represent an alternative to exercise. Several side effects were found, however, that may limit the potential utility of adenosine in CAD diagnosis, including severe chest pain, shortness of breath and flushing (49).

Ischemia/Reperfusion Injury - Ischemic damage occurs in tissues when an imbalance between oxygen demand and oxygen supply leads to a prolonged state of oxygen deficiency. Endogenous adenosine functions as a natural protectant of cells in ischemic tissue (50). Cells produce increased amounts of adenosine during ischemia as a consequence of an ischemia-induced net intracellular or extracellular ATP breakdown and as a result of their ability to divert ATP breakdown products to adenosine (51). Protection results from activation of extracellular adenosine receptors (38). Activation of A2a receptors localized on vascular smooth muscle cells leads to increased blood flow to the ischemic region by preferential vasodilation of the regional blood vessels (26). Activation of A2a receptors on platelets prevents platelet activation and subsequent platelet aggregation and therefore provides myocardial protection by inhibiting intracoronary thrombosis (28). An additional protective feature of adenosine arises from its stimulation of neutrophil A2 receptors, which prevents neutrophil activation, the adherence of neutrophils to the vascular endothelium and neutrophil-mediated production of cell-damaging oxidants such as hydroxyl radical (31). Neutrophils and neutrophil-derived mediators are key components of the inflammatory events that accompany myocardial ischemia (52).

The anti-ischemic properties of adenosine suggest that adenosine receptors represent potential drug targets for the treatment of acute myocardial infarction, angina, stroke and other conditions characterized by ischemia and reperfusion injury. Adenosine or adenosine agonists, however, provide inadequate protection in models of myocardial ischemia and, in fact, can increase the severity of the ischemia as a consequence of their ability to vasodilate normal vessels in preference to stenotic vessels (5). Furthermore, adenosine agonists are limited by their side-effect profile, which, as shown in man and in animals, includes angina pectoris-like pain, AV-block, hypotension, sedation and asthma (53).

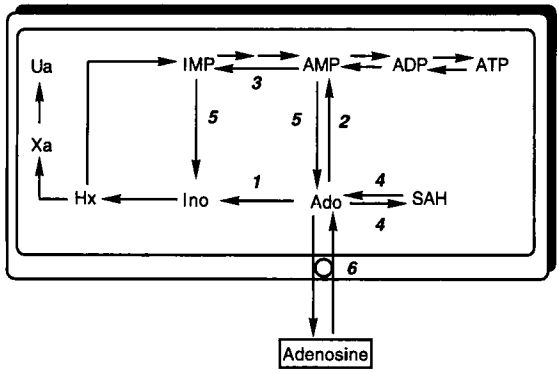
An alternative strategy that circumvents some of the limitations of adenosine agonists is to specifically increase endogenous extracellular adenosine levels in the ischemic region by enhancing adenosine production or by inhibiting adenosine utilization. These agents, now known as

"adenosine regulating agents" or ARAs, are pharmacologically silent except in tissue undergoing ischemia or some other condition that involves net ATP catabolism (5). The first compound identified with these properties was 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide or AICA riboside (**6**), which was shown to selectively increase adenosine levels in blood from ischemic regions of a canine heart and to have no cardiac or hemodynamic effects in non-ischemic regions (54). The site- and event-specificity of **6** results from its ability to increase extracellular adenosine concentrations only at sites of net ATP breakdown and from the short half-life of adenosine (48), which confines the pharmacological actions of adenosine to the site of production. Subsequent studies have further demonstrated the specificity of **6**. For example, **6** preserved cardiac function in canine hearts subjected to repeated episodes of oxygen demand induced ischemia as generated by atrial pacing in the presence of a coronary artery stenosis (55). Furthermore, **6** was shown to be effective in a model of myocardial stunning (56) and to have inhibitory effects on platelet aggregation *in vitro* (57). Recent Phase II and Phase III clinical studies in patients undergoing coronary artery bypass graft surgery have shown that **6** can decrease the frequency and severity of perioperative myocardial ischemia, including myocardial infarction and other adverse cardiovascular events (58).



6 **7** **8**

Other agents have been reported to protect the heart from ischemic damage by enhancement of extracellular adenosine. Several potential ARA targets exist based on the pathways and enzymes involved in adenosine production and utilization (Fig. 1). Adenosine deaminase (ADA) and the adenosine transporter represent two possible sites based on their role in adenosine metabolism. ADA inhibitors have been reported to be effective in models of global and regional ischemia. Two potent and selective ADA inhibitors, namely 2'-deoxycoformycin, **7**, and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), were found to improve recovery of contractile function in buffer perfused hearts undergoing global ischemia (59,60). In models of regional ischemia, EHNA was reported to increase extracellular levels of adenosine and to enhance postischemic regional function after transient reversible ischemia (61), while **7** was reported to be



1: Adenosine Deaminase
2: Adenosine Kinase
3: AMP Deaminase
4: SAH Hydrolase
5: 5'-Nucleotidase
6: Adenosine Transporter

Abbreviations: Ado: Adenosine; SAH: S-adenosylhomocysteine; IMP: Inosine Monophosphate; Ino: Inosine; Ua: Uric acid; Hx: Hypoxanthine; Xa: Xanthine

Figure 1. Adenosine Production and Utilization Pathways

effective in limiting ischemia-induced irreversible myocardial damage (62). Adenosine transport inhibitors have also been studied as potential cardioprotective agents. The classical transport inhibitor dipyridamole was found, however, to induce ischemia by provoking coronary steal in the presence of a coronary stenosis (63). Since coronary steal occurs at doses of dipyridamole known to inhibit phosphodiesterase, the mechanism of this effect is in question. Recently, a potent, orally active transport inhibitor R75231 (**8**) was shown to be cardioprotective in several *in vivo* models of myocardial ischemia, including dog hearts subjected to 1 hour of normothermic global ischemia, a cardiotransplant model and a model of myocardial infarction and cardiac failure (64).

Arrhythmia - In the heart, adenosine and adenosine agonists have profound electrophysiological effects in addition to their ability to vasodilate coronary vessels (33). Stimulation of A1 receptors located in the atrioventricular (AV) node and in the Purkinje fibers leads to a decrease in sinus node and Purkinje fiber automaticity and in AV nodal conduction (32). The net effect is bradycardia and a potential for life-threatening AV block. The short half-life of adenosine, however, has made these properties useful for the treatment of supraventricular tachyarrhythmias (SVTs) in humans (65). In these patients, adenosine was found to transiently block AV nodal conduction and thereby attenuate the abnormal heart rhythms without causing significant side effects.

Thrombosis - Agents that inhibit platelet aggregation have potential therapeutic utility in the treatment and prevention of a variety of cardiovascular and cerebrovascular diseases, including unstable angina, acute myocardial infarction, stroke and transient ischemic attacks (66). Activation of A2 receptors located on platelets by adenosine agonists leads to a potent inhibition of ADP- and thrombin-induced platelet aggregation (28). ARAs have also been shown to inhibit platelet aggregation. The transport inhibitors dipyridamole, 6-(*p*-nitrobenzyl)thioinosine (NBTI) and dilazep inhibited ADP-induced platelet aggregation in whole blood in the presence of adenosine (67). Pretreatment of plasma with the ADA inhibitor **7** prolongs the inhibition of platelet aggregation by adenosine (68). Adenosine kinase inhibitors, toyocamycin and sangivamycin, also show modest inhibition (69). More recently, **6** was shown to inhibit platelet aggregation *in vitro* (57) and to decrease intracoronary thrombosis *in vivo* (70).

CNS PHARMACOLOGY

Adenosine receptors are distributed throughout the brain and appear to be important in the modulation of CNS activity. A1 receptors are found predominantly in the hippocampus, neocortex, cerebellum and striatum, whereas A2 receptors are concentrated in the striatum, nucleus accumbens, olfactory tubercle and amygdala (71). Adenosine receptors are located on the pre- and postsynaptic membranes as well as on astrocytes and within the cerebral vasculature. Activation of A1 receptors leads to reduced neuronal activity. This effect arises from the ability of the presynaptic A1 receptor to inhibit excitatory neurotransmitter release and thereby reduce excitatory neurotransmission and from the ability of the postsynaptic A1 receptor to stabilize the membrane potential and thereby limit neuronal excitation (33). In contrast, adenosine antagonists such as caffeine are CNS stimulants presumably due to their antagonism of adenosine's inhibitory tone. The apparent role of adenosine in the brain and the pharmacology of adenosine receptor agonists and ARAs in a variety of CNS disorders, including stroke, epilepsy, brain trauma, anxiety, some psychoses, chronic neurodegenerative diseases and pain, has been reviewed (33,72-76).

Stroke - Neural tissue damage from prolonged cerebral ischemia and subsequent reperfusion occurs by the same mechanisms as found in the ischemic heart, *i.e.,* tissue acidosis, irreversible depletion of ATP and a loss of membrane integrity. Membrane damage, from *e.g.* lipid peroxidation, leads to abnormal ion homeostasis, membrane depolarization and cytotoxic edema (75). These events occur in the core ischemic region where blood flow has been severely impaired. In contrast, in the ischemic penumbra where collaterals can partially restore blood flow, mechanisms involving overactivation of the glutamate neurotransmitter system are more important (77). Damage produced by this mechanism, often termed "excitotoxicity", results from excessive release of the excitatory neurotransmitter glutamate followed by an overactivation of glutamate receptors located on the postsynaptic neuron. Overactivation leads to excessive influx of calcium which in turn initiates numerous pathologic events such as abnormal protease activation.

Activation of adenosine receptors protects neurons from ischemic damage by mechanisms similar to those described for the heart, *i.e.,* vasodilation to increase blood supply to the ischemic region, inhibition of platelet aggregation and inhibition of free radical production by granulocytes. In addition, endogenous adenosine produced during ischemia decreases damage to the penumbra by inhibiting glutamate-induced excitotoxicity (74,78). The adenosine inhibitory mechanism involves

activation of A1 receptors located on the pre- and postsynaptic membranes of excitatory neurons (33). Activation of the presynaptic A1 receptor inhibits glutamate release from the nerve terminal whereas activation of the postsynaptic A1 receptor inhibits the effects of glutamate receptor activation, including membrane depolarization, calcium influx and neuronal cell excitation. Presynaptically, A1 receptor activation is postulated to inhibit calcium influx through a voltage-sensitive calcium channel and thereby a calcium-dependent release of glutamate-containing vesicles from the nerve terminal. In contrast, the postsynaptic A1 receptor enhances potassium conductance, which inhibits excitation by limiting membrane depolarization (79). One advantage of adenosinergic compounds over glutamate receptor antagonists is that efficacy will be independent of the glutamate receptor subtype (NMDA, kainate, quisqualate) present on the postsynaptic neuron. Potential factors that could limit the overall efficacy of adenosinergic compounds, however, include glutamate release by A1 receptor-independent mechanisms, A1 receptor down regulation during ischemia and the timing of glutamate release relative to treatment.

Evidence that adenosinergic compounds are effective in reducing neuronal damage comes from studies in animal models of global and regional cerebral ischemia (74). Specific A1 agonists such as **1** are reported to reduce the damage to hippocampal CA1 cells after global ischemia (80). Another A1 agonist, **2**, is reported to reduce infarct volume in the cortex after focal ischemia (81). Studies to determine the mechanism of cerebroprotection are complicated by an A1 agonist-induced hypothermic response, which also provides protection (74). The therapeutic utility of A1 agonists as neuroprotectants is limited, however, by several additional A1 agonist-induced side effects, including hypotension, bradycardia and sedation. ARAs might circumvent some of the adenosine mediated side effects through elevation of adenosine levels in a site- and event-specific manner. Studies to date with adenosine transport inhibitors and ADA inhibitors have been inconclusive (75). For example, the transport inhibitor NBTI showed no protection in the gerbil ischemia model whereas the transport inhibitor propentofylline showed protection. Microdialysis studies of rats pretreated with propentofylline showed an increase in adenosine levels with a concomitant decrease in glutamate levels (82). In contrast, glutamate levels were said to be unchanged in rats pretreated with the ADA inhibitor **7**, despite an elevation of adenosine levels in cortical perfusates and some cerebroprotection (83,84). In other studies, **7** failed to inhibit neuronal damage caused by transient forebrain ischemia (85).

Seizure - Adenosine has been postulated to be an endogenous anticonvulsant, *i.e.*, a substance produced in the brain to control seizure activity (86). Support for the hypothesis comes from studies that show a dramatic rise in adenosine levels during a seizure and an increase in the spread and duration of electroshock, audiogenic and chemically-induced seizures after administration of the adenosine antagonist theophylline (for comprehensive reviews see 87,88). Consistent with this hypothesis are studies that show that adenosine agonists decrease the severity of seizures in a dose-dependent manner by shortening the duration and limiting the propagation of the seizure. For instance, **2** protected rats in a theophylline-reversible fashion from seizures induced by pentylenetetrazol (PTZ). In amygdala-kindled seizures, both the A1 agonist **1** and the non-selective agonist **4** exhibited a dose-related caffeine-reversible reduction in seizure severity and afterdischarge following intraventricular administration. These studies as well as others indicate that A1 receptor activation is responsible for the anticonvulsant effect (87). The mechanism of the anticonvulsant effect is clouded, however, by the multitude of pharmacological activities elicited by A1 agonists, *e.g.*, hypotension, sedation, muscle relaxation and hypothermia. Several studies indicate that these activities cannot account for the anticonvulsant activity. For example, the time course of sedation does not correlate with the anticonvulsant effects. Furthermore, nonadenosinergic compounds that produce hypotension or muscle relaxation fail to protect mice from PTZ seizures. In contrast, the effects of A1 agonist on excitatory neurons are at least consistent with the observed anticonvulsant activity. A1 receptor activation on the presynaptic neuron inhibits glutamate release and overexcitation whereas A1 receptors on the post synaptic neuron increases potassium conductance. Increased potassium flux stabilizes the membrane potential and thereby decreases the ability of the neuron to depolarize, which in turn limits nerve cell excitability and repetitive firing (33).

The cardiovascular effects of A1 agonists may limit their utility in the treatment of epilepsy. ARAs appear to be a viable alternative given that they are hemodynamically silent and are specific for sites undergoing ATP degradation (*e.g.* ischemia, seizure). Several studies have reported positive results for ARAs in seizures. The adenosine kinase inhibitors, 5'-amino-5'-deoxyadenosine and 5-iodotubercidin, are reported to suppress bicuculline methiodide-induced seizures after injection into the prepiriform cortex (89). In the same study, transport inhibitors NBTI and dilazep were slightly less effective and the ADA inhibitor **7** had only weak activity.

Pain - Excitatory amino acid neurotransmission has been implicated in chronic neuropathic pain (90). Activation of A1 and possibly A2 receptors located on neurons in the spinal cord is reported to suppress neurotransmission and nociception (34). A1-selective agonists **1** and **2** and a non-selective agonist **4** are reported to produce antinociception in standard pain models. Interestingly, some studies indicate that the spinal analgesia produced by morphine may result from a morphine-induced release of adenosine from nerve terminals (91). An ARA approach to pain has also yielded positive results. For example, the adenosine kinase inhibitor 5'-amino-5'-deoxyadenosine was efficacious in the tail flick model after intrathecal administration (92). The ADA inhibitor **7**, however, was ineffective.

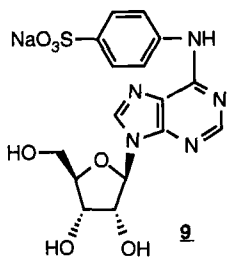
OTHER PHARMACOLOGY

Inflammation - Adenosine is believed to function as an endogenous antiinflammatory agent (29) based on its ability to inhibit cellular adhesion (30) and superoxide anion generation (31). Tissue damage induced by inflammation stems in part from the migration of neutrophils to the sites of inflammation (*e.g.*, sites where there is infection, tissue injury, ischemia or complement activation) followed by the release of membrane-damaging oxygen radicals. Adenosine inhibits both activities (30,31) and therefore could be important in limiting abnormal and excessive inflammatory reactions, such as those found in rheumatoid arthritis, osteoarthritis, psoriasis and inflammatory bowel disease.

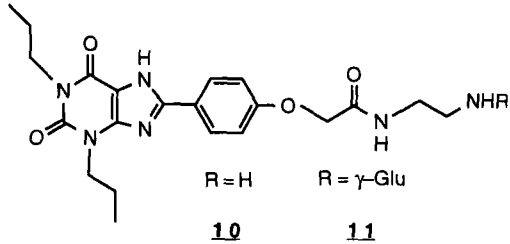
The adenosinergic antiinflammatory mechanism is still under investigation but appears to center on the immunoregulatory activity of adenosine (29). For example, neutrophil migration is inhibited by activation of neutrophil A2 receptors based on *in vitro* studies, which showed the A2 agonist **4**, but not the A1 agonist **1**, to inhibit in a dose-dependent manner adhesion of fMLP-stimulated neutrophils to endothelial cells (30). Selective activation of A2 receptors by **5** was also shown to inhibit oxygen free radical production (31). In contrast, activation of A1 receptors on the neutrophil is reported to stimulate inflammatory events such as chemotaxis and phagocytosis (31). Interestingly, some *in vivo* studies have shown that A1-selective agonists are more efficacious antiinflammatory agents than A2 agonists (93,94). For example, the A1 agonist **2** is reported to partially reverse the decrease in circulating neutrophils after intrapleural injection of the proinflammatory agent carrageenan to rats (93). These results suggest that A1 receptors might mediate in some manner neutrophil adhesion and migration into tissues. Other antiinflammatory effects of **2** were noted in these studies, including a decrease in the exudate volume and a weak inhibition of superoxide production.

An adenosinergic mechanism has recently been postulated to account, at least in part, for the functional benefit frequently obtained in rheumatoid arthritis patients given low doses of the dihydrofolate reductase inhibitor methotrexate (MTX) (95). In cell culture, MTX is reported to increase adenosine release and to inhibit neutrophil adhesion to fibroblasts. The ARA, **6**, had similar effects leading to the hypothesis that polyglutamated MTX, which accumulates in inflammatory cells and is a potent inhibitor of AICAR transformylase, enhances adenosine release by increasing the endogenous production of **6** or its corresponding 5'-monophosphate.

Diabetes - Hepatic glucose output is reported to correlate with the high fasting blood glucose levels found in type II diabetics (96). Since gluconeogenesis, *i.e.* the biosynthesis of glucose from low molecular weight precursors (*e.g.* lactate), appears to be the major pathway responsible for glucose production (97), several approaches have been taken to either directly or indirectly inhibit gluconeogenesis. One approach is to starve the liver of free fatty acids (FFAs). FFAs undergo β-oxidation to produce higher levels of acetyl-CoA and NADH in the liver, both of which accelerate gluconeogenesis (98). FFAs are produced by lipolysis of stored triglycerides in adipocytes. Type II diabetics have increased lipolysis activity and elevated levels of the products of lipolysis, *i.e.*, glycerol and FFAs (99). Consequently, A1 agonists are potentially useful for the treatment of diabetes, since activation of the A1 receptor on adipocytes leads to potent inhibition of lipolysis (35). In man, treatment with adenosine receptor antagonists results in increased lipolysis, suggesting that basal levels of adenosine have an inhibitory effect on lipolysis (100). Other studies that support the use of A1 agonists in diabetes, include the finding that A1 agonists such as **2** decreased lipolysis in adipocytes from diabetic animal models (101) and the report that a water soluble peripherally-acting A1 agonist, **9**, dramatically suppressed serum glycerol levels in rats at a dose of 0.1 mg/kg s.c. (102). A clinical study of an A1 agonist is reported to be underway (103).



9



10 11

Renal Failure - Endogenous adenosine is postulated to be an important regulator of renal function (36). Much of the support for this hypothesis comes from data on non-selective adenosine antagonists, such as theophylline, which have been shown to increase renal blood flow, increase glomerular filtration rate, induce diuresis and natriuresis and enhance renin secretion. These properties are apparently mediated by the A1 receptor based on the similar pharmacology produced by A1-selective antagonists and based on the location of A1 receptors in the kidney (juxtaglomerular cells, afferent arterioles, tubular cells) (104). Acute renal failure is characterized by elevated renal adenosine levels and by decreased renal blood flow and glomerular filtration rate. Hence, efforts have been made to find potent and selective A1 antagonists for the treatment of renal failure. Numerous A1-selective antagonists have been described (105,106) and shown to be protective in models of acute renal failure (107). Furthermore, the potent A1 antagonist **10** is reported to be generated selectively in the kidney from the prodrug **11** by the kidney enzyme γ-glutamyl transferase (108).

Conclusion - Adenosine and adenosine receptors are associated with a multitude of pharmacological activities. Consequently, few adenosinergic compounds have been developed to date due to the difficulty of finding compounds that produce beneficial pharmacology without severe, or at least limiting, side effects. Recent cloning efforts, however, indicate that there are many more adenosine receptor subtypes than originally thought thereby leaving open the possibility that subtype-specific compounds will elicit more specific pharmacological responses. Furthermore, ARAs are expected to be clinically useful based on their ability to attain pharmacological specificity through elevation of adenosine levels in a site- and event-specific manner. Future efforts that further define adenosine receptor structure-function, adenosine production and metabolic pathways and methods for tissue-specific delivery of prodrugs may enable the successful development of adenosinergic compounds for the treatment of a variety of diseases.

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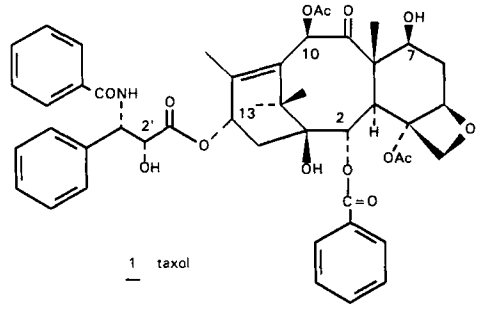
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Chapter 32. Taxol: From Discovery to Therapeutic Use

Matthew Suffness
Developmental Therapeutics Program, Division of Cancer Treatment
National Cancer Institute, NIH, Bethesda, MD 20892

Introduction - There is a continuing need for development of new anticancer drugs, drug combinations and chemotherapy strategies (1). Natural products have been a fertile source of novel chemotypes for anticancer research. This chapter provides the first review in this series on taxol (**1**), an agent first reported in 1971 (2), which has been found beneficial in treatment of refractory ovarian, breast, and other cancers and was recently approved for marketing. Taxol provides both a novel chemotype and a novel mechanism, stabilization of microtubules, for cancer therapy. However, the clinical development of taxol has been difficult due to poor solubility, supply problems, and hypersensitivity reactions. Previous reviews of taxol have covered the chemistry (3-6), approaches to total synthesis (7-9), supply (10), antitumor activity (11,12), mechanism of action (13-16), and clinical development (17-21). This chapter summarizes the discovery, development, clinical status, approaches to supply and structure-activity relationships in the taxol area.



1 taxol

DISCOVERY

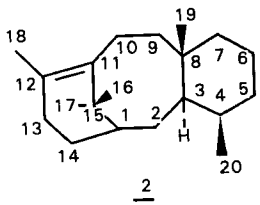
To spur development of new cancer drugs, a screening and discovery program for cancer chemotherapeutics was established at the National Cancer Institute (NCI) in 1960. Screening of plant extracts began with a survey of the flora of the U.S. conducted in collaboration with the U.S. Department of Agriculture (USDA) (22,23). *Taxus brevifolia* Nutt. (Family Taxaceae), the Pacific yew or Western yew, was collected in 1962 as part of this program from Washington State. The Pacific yew is a smallish, slow growing tree native to the Pacific Northwest with a North-South range from Southeastern Alaska to Northern California, and extending eastward to mountainous areas of Idaho and Montana. It is often found as an understory tree in populations of Douglas fir. The Taxaceae is a small, somewhat isolated, botanical family with 5 genera of which *Taxus* is the most prominent with eleven species worldwide.

Isolation of Taxol - The first activity of *T. brevifolia*, cytotoxicity to KB cells (derived from a nasopharyngeal tumor), was confirmed in 1964. *In vivo* antitumor activity in the Walker 256 carcinosarcoma, P1534 leukemia, and L1210 leukemia models was later observed. The lead was assigned to Dr. Monroe Wall's group at the Research Triangle Institute (RTI), under contract to the NCI, in 1965. The isolation of the pure active substance, guided by cytotoxicity assays against KB cells and *in vivo* assays in the P1534 leukemia, was achieved in 1966. The isolated yield from dried bark of Pacific yew was only 0.02%. The molecular formula was elucidated as $C_{47}H_{51}NO_{14}$ and data from the ^{1}H-NMR spectrum indicated that the compound was a diterpenoid possessing the uncommon taxane skeleton (**2**), until now only found in the family Taxaceae. Mild methanolysis of **1** gave a tetraol (**3**) which was characterized by X-ray crystallography as its bisiodoacetate (**4**) and a methyl ester derived from the C-13 side chain (**5**) which was characterized as its p-bromobenzoate (**6**), also by X-ray

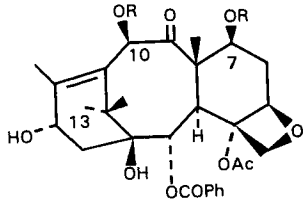
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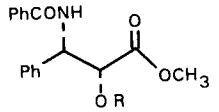
crystallography. The total structure of **1** was deduced from the combination of the X-ray and ^{1}H-NMR data (2). It is noteworthy that the bisiodoacetate **4** formed from tetraol **3** rather than the trisiodoacetate, providing a first indication of the difficulty in esterification of the secondary alcohol at C-13 that was later to be a significant problem in semisynthesis of taxol and analogues.



2



3 R = H
4 R = COCH$_2$I



5 R = H
6 R = -p-C$_6$H$_4$Br

DEVELOPMENTAL STUDIES

Antitumor activity - In the early 1970s, taxol was not considered to be a viable development candidate. The only tumor system showing strong activity, the P1534 leukemia, was not thought by the NCI to be predictive of potential clinical activity. Moreover, the activity seen against the L1210 and P388 leukemia models was very modest compared to that of the synthetic antimetabolites and alkylating agents that were considered the best clinical agents of the period. The very poor solubility of taxol and the limited supply prospects were not encouraging. In 1974-75, the murine B16 melanoma model, newly introduced to the NCI screen, was found to be highly sensitive to taxol (life extensions >150%) and this led to the selection of taxol as a preclinical development candidate in 1977. Contemporaneously, the athymic (nude) mouse became available in sufficient quantities for antitumor experiments, thus enabling the NCI to set up a "tumor panel experiment". The activity of clinical agents, developmental agents and newly identified P388 leukemia prescreen active agents was compared against standard murine tumors *versus* human tumors carried as xenografts in the athymic mice (24). Taxol caused regression of the MX-1 human mammary tumor, and showed moderate activity with growth inhibition of 70-90% of the LX-1 lung tumor and the CX-1 colon tumor, the only other human tumors in the experiment (12). The overall findings for the 500 compounds tested in the "tumor panel experiment" were that most compounds tested were considerably more active against the murine than the human tumors, and that the xenografts as a whole were relatively drug resistant. Taxol exhibited a highly unusual profile, showing better activity against the human tumor xenografts than the murine tumors. Other xenograft studies have found taxol active against human hepatic metastases of breast cancer, cutaneous metastases of bronchial cancer, a tumor of the base of the tongue, and an ovarian tumor (25,26). Taxol has higher activity against ovarian xenografts HOC18 and HOC22 than cisplatin (27) and when formulated and administered i.v.,it is quite active against the OVICAR-3and -5 ovarian, the NCI-H522 and ASC EKVXnon-small cell lung, and the HL-60 leukemia xenografts (28). Likewise, significant cytotoxicities at levels achievable in plasma, have been found for taxol against freshly explanted ovarian carcinomas (29,30). The antitumor activity of taxol in experimental models indicate that taxol has better activity against solid tumors than against leukemias (11,12). Recent combination studies of taxol with cisplatin, doxorubicin, etoposide and cyclophosphamide have not shown superiority to the single agents (11). The sequence of administration in combinations can be critical for taxol activity; when taxol is given before cisplatin the combination is more active and less toxic (31). Taxol sensitizes human astrocytoma cells to radiation due to antimitotic activity, causing cells to accumulate in the G_2 and M phases of the cell cycle where they are more radiosensitive (32). Cell lines having the multi-drug resistance (MDR) phenotype which are known to express large amounts of the membrane phosphoglycoprotein MDR-1, are resistant to taxol, indicating that the drug is removed from cells by the ATP-driven MDR pump (15,33,34). Taxol-resistant Chinese hamster ovary cell lines with specific tubulin mutations have been characterized (35,36), while the Tax-18 cell mutant is taxol-dependent (37). This last cell line is useful to assay for taxol-like activity.

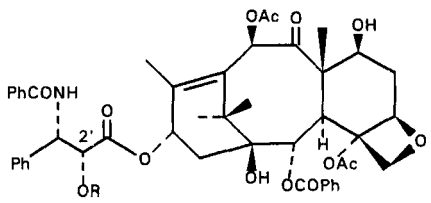
Mechanism of Action - The first mechanistic study, reported in 1978, found that taxol was a mitotic spindle poison (38). Shortly thereafter, the surprising observation was made that. unlike all of the mitotic inhibitors known to that time (e.g. colchicine, vincristine, podophyllotoxin, maytansine), taxol did not bind to tubulin monomers and prevent assembly into microtubules, but rather it accelerated the polymerization of tubulin and stabilized microtubules (39-41). Additional studies showed that the rate enhancement for assembly of tubulin resulted from a shift in equilibrium towards the microtubules, stabilizing them against depolymerization back to α- and ß-tubulin subunits. Taxol-induced microtubules are very resistant to depolymerization by $CaCl_2$ or cold (0°), suggesting that the taxol binding site is on microtubules rather than tubulin (13-16,42). Taxol effects were not competitively inhibited by tubulin binders, (vincristine, colchicine, or podophyllotoxin), indicating a different binding site. Displacement studies with ^{3}H-taxol (43) or ^{3}H-7-acetyl taxol (44), established binding at a site on the pre-formed microtubule. Photolysis of bound ^{3}H-labelled taxol, followed by depolymerization, specifically labelled the ß-tubulin subunit (45). These results indicated the binding is reversible and the binding site is on the microtubule at a place in the amino acid sequence derived from ß-tubulin. Photoaffinity-labelled taxol derivatives have been synthesized, including a p-azido moiety on either the C-3' benzamide (46), or a C-7 benzoyl ester (47), and a compound having a benzoyl trifluoromethyl azirine at C-7 (48). No results of tubulin labelling and degradation experiments with these compounds have been reported.

The mechanism of vincristine, vinblastine, and colchicine as antitumor agents is fairly clear - they inhibit assembly of tubulin into microtubules and block formation of the mitotic spindle and hence prevent cell division. These agents are most effective against rapidly dividing cells and, in particular, show strong activity against mouse leukemia models. The case is not so clear for the antitumor mechanism of taxol for two main reasons: a) there are microtubule bundles, fibers, and asters which are found in many parts of the cell following taxol treatment (49-51) and, for example, microtubules are known to function in the cytoskeleton, in secretion, in transport, and cellular motion indicating possible alternate mechanisms; b) the relatively superior antitumor activity against solid tumors compared to leukemias in experimental models, combined with the clinical activity against ovarian and breast cancers, present a quite different profile than would be expected for an agent working primarily by the mechanism of blocking cells in mitosis. Other studies for example, indicate that taxol influences the tyrosine phosphorylation of microtubule-associated proteins (MAPs), stimulates MAP-2 kinase activity (52-53), and induces secretion of TNF-α (54,55). Further investigations are needed to determine how taxol works as an antitumor agent.

Formulation - Taxol is classified as highly insoluble in water (56). The optimal antitumor dose in mice is 10-40 mg/kg for either the i.p. or i.v. route depending on the tumor model and frequency of administration (11,12,25,28). The target formulation concentration for i.v.administration in humans was >5 mg/ml, giving a preparation stable for more than 24 hours after dilution into a standard i.v. fluid such as 0.9% saline or 5% dextrose in water. A large number of formulations were investigated including true solutions, emulsions, micelles, and surfactant preparations. The final product chosen for development contained 30 mg of taxol in 5 ml ampules with 50% Cremophor EL (polyethoxylated castor oil) and 50% dehydrated alcohol. The concentration of Cremophor EL delivered was considerably higher than that in any marketed product using this surfactant and Cremophor EL is known to cause histamine release and to be a potential clinical problem due to allergic reactions (57-58).

Prodrugs of taxol have been prepared by placing solubilizing moieties at the 2'-hydroxyl group on the side chain including succinate and other diacid derivatives (**7,8**), taurine related derivatives (**9,10**), and amino acid derivatives (**11,12**) (59-62). Esters at the 2'-hydroxyl are quite reactive and undergo chemical hydrolysis readily. Since trace hydrolysis gives rise to the highly insoluble taxol, these prodrugs have not proven chemically stable enough for development. Prodrugs that are enzymatically activated may be a more successful approach. Liposomal formulations of taxol are reported to have better activity than the parent drug against gliosarcoma and glioblastoma xenografts (63), but nanocapsules are more toxic than the free drug (64). A 50% triacetin emulsion of taxol showed good pharmaceutical stability (65).

Toxicology - Taxol was administered by the intraperitoneal route to Sprague-Dawley rats in both single doses and in daily doses for 5 consecutive days. The LD_{50} was 206 mg/m^2 on the single dose schedule and 51 mg/m^2/day on the 5 day schedule. The same 5-day schedule in CD2F$_1$ mice showed



7 R = $-CO(CH_2)_3CONH(CH_2)_3N(CH_3)_2$ HCl

8 R = $-COCH_2CH_2NH_3$ $HCOO^-$

9 R = $-COCH_2CH_2CONHCH_2CH_2SO_3^-$ Na^+

10 R = $-COCH_2CH_2SO_3^-$ Na^+

11 R = $-COCH(CH_3)NH_2$

12 R = $-COCH_2N(CH_3)_2$

an LD_{50} of 82 mg/m^2/day (17). Toxicity was also evaluated in beagle dogs by the intravenous route. In all three species, typical toxicities for antiproliferative drugs were noted including effects on the hematopoietic, gastrointestinal, lymphatic and reproductive systems. These data suggested that myelosuppression would be the dose limiting toxicity (DLT) in human trials. The Cremophor vehicle control as a large volume single dose in dogs produced hypotension due to vasodilation, followed by lethargy and death. The vehicle was better tolerated on repeated doses of smaller volumes, and did not show evidence of cumulative toxicity. Dogs are more sensitive to histamine release by Cremophor than other species, but these data suggested a potential for hypersensitivity problems in the clinical trials (17). Toxicology studies were completed in 1982 and the IND application was filed by the NCI in 1983.

CLINICAL TRIALS

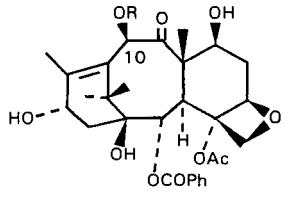
Phase I Trials - Initial trials were conducted in 1983 on multiple schedules of administration (17,21). DLTs for seven of nine trials were neutropenia, consistent with the toxicology predictions, with the other two DLTs being mucositis and hypersensitivity reactions (HRs). Most trials reported HRs and some neuropathies were seen, mainly as partial paralysis and numbness of the extremities. The recommended Phase II doses were in the range of 210-250 mg/m^2 on the single infusion schedules and 30 mg/m^2/day on the 5-day schedule. Partial or minor responses were seen in patients with melanoma, ovarian, head and neck, and non-small cell lung cancer. The DLT, hematologic toxicity, is consistent among patients, with neutrophil counts recovering within the 21 day period between courses of therapy, but this hematologic toxicity is not cumulative. The HRs were more serious however, as they included several cases of severe anaphylactoid reactions. Evaluation of these reactions across several trials found that the incidence was higher on the shorter infusion schedules (possibly related to peak Cremophor concentration) and that the reactions were likely histamine induced, leading to recommendations for Phase II trials being conducted on a 24-hour infusion schedule with premedication including diphenhydramine, dexamethasone, and cimetidine (58). This has been largely effective in reducing the incidence of HRs in Phase II trials (21). Cardiac toxicity, mainly in the form of asymptomatic bradycardia, has been noted in many trials, but its significance is not known (66).

Phase II Trials - Additional trials of taxol were hampered by lack of drug supply, and early trials were limited to ovarian, melanoma, colon, cervix, prostate and renal cancers. The impetus to further development was a study reported in 1989 showing a 30% response rate in refractory and advanced ovarian cancer (67), which was confirmed in two subsequent trials (68,69). An NDA was filed for the indication of refractory ovarian cancer in July, 1992 and was approved by the FDA in December, 1992. Important activity has been observed in metastatic breast cancer with 56% and 62% response rates reported from two studies (21,70,71). Activity in Phase II trials has also been observed in non-small cell lung cancer and head and neck cancer prompting expansion of such trials (21). The full spectrum of activity of taxol remains unknown, since Phase II trials in many disease states are still too early to evaluate . Recovery from the main DLT of taxol, myelosuppression, can be accelerated by concurrent use of granulocyte colony stimulating factor (G-CSF) (72). One such study in refractory ovarian cancer using taxol and G-CSF has increased doses from 170 mg/m^2 to 250 mg/m^2 with an increase in response rate to 50% from the approximately 30% rate without G-CSF support (72). Combination studies with standard agents, such as taxol with cisplatin in ovarian cancer, and taxol with either doxorubicin or cyclophosphamide in breast cancer, are underway (21).

CHEMISTRY

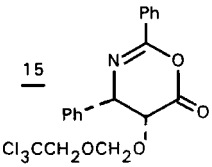
Natural Abundance - The reported yield of taxol from Wall's original isolation from Pacific yew (*T. brevifolia*) bark was 0.02%, although typical yields on large scale collections are below 0.01%. One large tree yields enough drug for one treatment for one patient. Since the yew is very slow growing, and bark collection kills the tree, it quickly became apparent that supply needs could not be met from natural harvest over the long term. *Taxus* species have been investigated world wide including the European yew (*T. baccata*), Japanese yew (*T. cuspidata*), Canadian yew (*T. canadensis*), Chinese yews (*T.mairei, T. yunnanesis, T. chinensis*), and the Himalayan yew (*T. wallichiana*) without finding a source of taxol superior to Pacific yew bark, although needles of some species and ornamental crosses are comparable (74,75). Results depend greatly on harvest and drying protocols, especially for needles, where the taxol content seems unstable. Seedlings have been shown to contain levels of taxol similar to bark, suggesting planting of seedling crops, as well as harvesting plantations of ornamental yews, particularly *T. X media* var. Hicksii, as promising sources (74,75). Plant cell culture production of taxol has been achieved by several companies, and this could become a commercially viable source of taxol if high enough titers can be achieved,.

Semisynthesis - There are two naturally occurring compounds found in yew, similar to taxol but lacking the C-13 side chain, baccatin III(**13**) and 10-desacetylbaccatin III(DAB; **14**). DAB is found in needles and twigs of European yew at a concentration about ten times that of taxol in Pacific yew (76). Since needles and twigs may be obtained from clippings, this is a renewable source of taxol precursors. The main difficulty lies in the esterification of C-13, since taxol, baccatin III, and DAB are all cup-shaped (77) and the C-13 hydroxyl group is very sterically hindered. Furthermore, the taxol side chain contains two bulky phenyl groups. Sophisticated blocking group chemistry is also needed for the hydroxyls at positions 7, 10, and 2' in the side chain. The first semisynthesis involved esterification of C-13 with a cinnamoyl group, and used the double bond to introduce the C-2' and C-3' substituents via hydroxyamination (78). The first practical synthesis coupled the intact, protected side chain to a protected baccatin III and gave an overall yield of 38%, assuming no recycling (79). Significant yield improvements have been made through the use of more compact groups such as the oxazoline (**15**) and the ß-lactam (**16**) to deliver the side chain carbonyl to the C-13 hydroxyl of protected DAB or baccatin III (80,81). Taxol is now produced in yields of 85-90% from addition of protected side chain to C-13 of DAB, with deprotection steps included. Hundreds of kilos per year of DAB are now being extracted from European yew and the semisynthetic ß-lactam process will be the source of most taxol starting in 1994 (82). Several highly stereospecific and short syntheses of the taxol side chain are available (83-88). Given the relative abundance of DAB from needles of *T. baccata* (a renewable resource) and the improvements in methodology for both the side chain synthesis and the coupling to C-13, a continuing supply of taxol is now assured. However, a better source of baccatin III, DAB, or taxol itself from either agricultural, plant cell culture, semisynthetic or total synthesis approaches remains highly desirable.

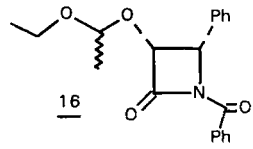


13 R = Ac baccatin III

14 R = H 10-DAB



15



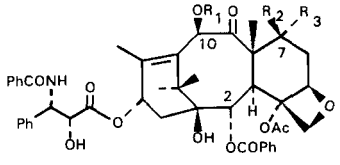
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Total Synthesis - Given the efficient chemistry of the side chain synthesis and the coupling reactions developed above, the total synthesis of taxol is reduced to a synthesis of either baccatin III(**13**) or DAB(**14**). The main synthetic problems are the nine asymmetric centers present in the core structure, the synthesis of the eight-membered B ring, the introduction of the oxetane D ring, and the stereospecific introduction of substituents at positions 1,2, 4,5,7,10, and 13 along with the protecting group chemistry necessary to permit differentiation of the hydroxyl and acyl substituents. The

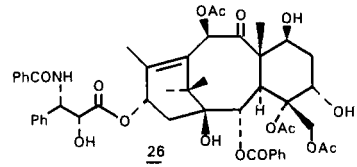
synthesis of taxanes has been a daunting task and to date only one synthesis of a natural taxane has been completed, that of the enantiomer of taxusin, a considerably simpler molecule than taxol which has both fewer substituents and lacks the oxetane ring (89). Synthetic approaches to the taxanes comprise an extensive literature; for example the key 8-membered B ring has been made by direct cyclization, transannular cyclization of larger rings, 4 + 4, 4 + 2, or 3 + 3 cycloadditions or sigmatropic rearrangements, ring expansions by photocycloadditions and cyclobutane openings by fragmentation reactions (3,4,7,8,9). One of the most promising approaches uses pinene as a starting material to give the taxane ring system in 5 steps (90). While an efficient total synthesis is highly desirable, another key role of total synthesis will be to prepare taxanes with specifically deleted substituents and altered stereochemistry to study structure-activity relationships. The ultimate goal of these efforts is a much simplified compound with taxol-like activity.

Structure-Activity Relationships (SAR) - For convenience, the SAR of taxol can be divided into three areas: the North (positions 7,9,10 and the 11-12 olefin); the South (positions 1,2,4 and 5); and the C-13 side chain. There are three levels of assays which have been used in SAR studies: at the biochemical level, using assays for either polymerization of tubulin or inhibition of depolymerization of microtubules; at the cellular level, measuring cytotoxicity to tumor cell lines such as KB, J774.2 macrophages, P388 leukemia, or B16 melanoma; and at the organism level, examining growth inhibition of either mouse tumors or human tumor xenografts. The correlation between mammalian tubulin assays and cell culture assays is generally good, except for some highly polar analogues that fail to penetrate cells efficiently. Some polar compounds that are effective in cell culture have not been effective in tumored animals, perhaps due to rapid excretion or metabolism to inactive forms.

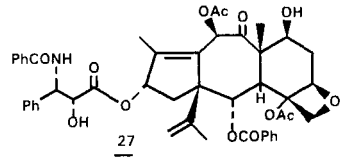
SAR - the North - In J774.2 cells, 7-epitaxol (**17**), 7-acetyl taxol (**18**), 10-desacetyltaxol (**19**), and 10-desacetyl-7-epitaxol (**20**), are all 1.5- to 4-fold less cytotoxic than taxol (5,91,92). 7-Xylosyltaxol (**21**), 10-desacetyl-7-xylosyltaxol (**22**), and 7-glutaryltaxol (**23**) have similar tubulin activity as taxol (93,94). 7-Amino acid substituents including 7-N,N-dimethylglycyltaxol (**24**) and 7-L-alanyltaxol (**25**) were about half as cytotoxic as taxol in B16 cells, but **24** was considerably less active than taxol against the MX-1 mammary xenograft in vivo (61). Taken together, these data indicate that acylation or deacylation at positions 7 and 10 lack major impact on taxol activity and that polar, and even bulky substituents are tolerated on the 7-hydroxyl, albeit with some loss of *in vivo* activity. However, conversion of either the 7- or 10- hydroxyl to ketones results in decrease of cytotoxicity to KB cells by $>10^3$; loss of activity of the 7-keto derivative may be due to facile ring opening to form a D-seco compound (48).



CMPD	R-1	R-2	R-3
17	Ac	H	OH
18	Ac	OAc	H
19	H	OH	H
20	H	H	OH
21	Ac	O-Xylosyl	H
22	H	O-Xylosyl	H
23	Ac	$-OCO(CH_2)_3COOH$	H
24	Ac	$-OCOCH_2N(CH_3)_2$	H
25	Ac	$-OCOCH(CH_3)NH_2$	H



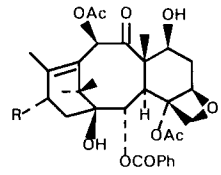
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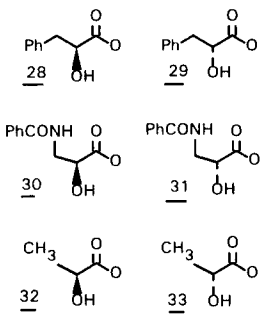


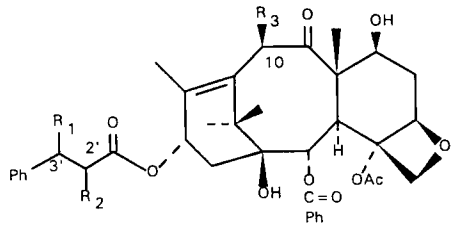
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SAR - The South - This is the least studied region of taxol, largely due to the chemical and steric inaccessibility of the functionalities present. No SAR have been reported on C-1; only one change at C-2 is known, the metabolic hydroxylation in rat bile of the benzoate ester to m-hydroxybenzoate which had similar tubulin assembly activity but was 39-fold less cytotoxic to L1210 cells (95). The oxetane is generally quite stable but treatment of taxol with triethyloxonium tetrafluoroborate gave **26** which is $>10^5$ less cytotoxic. Mesylation of a protected taxol led to ring A rearrangement product **27**, which was 100-fold less cytotoxic to KB cells but which retained tubulin polymerization activity similar to taxol (96).

SAR - C-13 Side Chain - This is both the most investigated and the most critical part of taxol for activity recognized to date. Baccatin III(**13**) is 50-fold less active than taxol in a mammalian tubulin assay, and a series of baccatin III derivatives are even less active, firmly establishing the need for an ester at C-13 (91,93). The minimal side chain requirements for activity have been tested by deletion of the 3'-benzamide (**28,29**), the 3'-phenyl (**30,31**), and both 3' substituents (**32,33**). The most active compounds were **28** and **29** which however were much slower and less potent than taxol in tubulin assembly and had EC_{50} values 15-30 times higher than taxol in CHO and J774.2 cells (97). Compounds **30** and **31** are less active in both the tubulin and cytotoxicity assays as a result of altered conformation of the side chain; natural stereochemistry at C-2' is desirable (97). An extensive SAR study of inhibition of microtubule disassembly, in which the 3'-phenyl is retained, examined effects of both substituents and stereochemistry at 2' and 3' (94). Deletion of both 2' and 3' substituents (**34**) or the 3'-benzamide only (**35**) decreased potency. 10-Desacetyltaxol (**19**) is almost as potent as taxol (**1**); taxol becomes 10-fold less active on reversal of 2' and 3' substituents (**36**) and over 100 fold less active when the stereochemistry is also reversed as in **37**. Reversal of 2',3' stereochemistry in **1** gives **38**, 4.5 times less active. A free amino group at 3' (**39**) is much less active than the benzamide in **1**, but the amino group can be acylated by a tigloyl ((E)-2-methyl-2-butenoyl) group as in the naturally occurring cephalomannine (91), the tosyl group as in **40**, or the t-butyloxycarbonyl moiety as in **41** and **42** without much change in activity (94). Other substitutions on the 3'-benzamide including p-hydroxylation or p-chlorination have little effect on tubulin activity but may decrease cytotoxicity (95,98). Reversal of stereochemistry to 2'S,3'R in **42** gives **43** which is 60-fold less potent (94). Esters at the 2'-hydroxyl decrease tubulin activity, particularly bulky or inactivated esters (60,91). Compound **42** (Taxotere®, RP 56976) has recently been advanced to clinical trials in both the US and Europe based on increased potency, only partial cross-resistance with taxol, and a formulation which uses Tween 80 rather than Cremophor (99-111). Encouraging responses have been seen in Phase I trials (108-111).

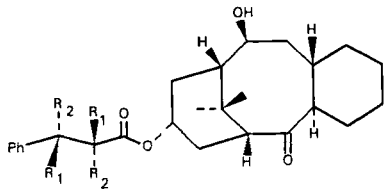






CMPD	STEREO	R_1	R_2	R_3	ED50 / ED50-TAXOL
1	2'R,3'S	NHCOPh	OH	Ac	1
19	2'R,3'S	NHCOPh	OH	H	1.3
34	----	H	H	H	17
35	2'R	H	OH	H	4.5
36	2'R,3'S	OH	NHCOPh	Ac	10
37	2'S,3'R	OH	NHCOPh	Ac	110
38	2'S,3'R	NHCOPh	OH	Ac	4.5
39	2'R,3'S	NH_2	OH	Ac	44
40	2'R,3'S	NH-tosyl	OH	H	5
41	2'R,3'S	NHCOO-t-Butyl	OH	Ac	0.5
42	2'R,3'S	NHCOO-t-Butyl	OH	H	0.5
43	2'S,3'R	NHCOO-t-Butyl	OH	H	30

SAR Models - The above data and molecular modelling and NMR studies lead to the conclusion that the C-13 side chain may interact in a hydrophobic cleft on mammalian microtubules needed for



44 R_1 = OH, R_2 = H
or R_1 = H, R_2 = OH

binding and that the side chain stereochemistry should be the natural one for best activity (94,97). The caged nature of the taxane ring results in interactions of the side chain in **42** with the 18-methyl and the 4-acetate (77,112). There may be a significant interdependence between side chain substituents and stereochemistry and ring substituents which will complicate the SAR analysis. The oxetane and the 2-benzoate may be important in locking in the active conformation (94,96,97,112). Additional analogues and additional modelling and NMR experiments are needed to define the pharmacophore. The only totally synthetic compound reported to show inhibition of tubulin polymerization is **44**, but no quantitative data were reported (113).

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Chapter 33. Recent Advances in the Generation of Molecular Diversity

Walter H. Moos and Grant D. Green
Chiron Corp., 4560 Horton Street, Emeryville, CA 94608

Michael R. Pavia
Sphinx Pharmaceutical Corp., 840 Memorial Drive, Cambridge, MA 02139

Introduction - Generation and screening of molecular diversity has become a major tool in the search for novel lead structures. This field was the subject of two previous chapters in this series on natural products (1), and on recombinant and synthetic "randomized" peptide libraries (2). Other recent reviews address new drugs from natural sources (3), biodiversity in biotechnology (4), multiple peptide synthesis (MPS) methods and applications (5), and the generation of molecular diversity (6,7). The present chapter provides a comprehensive review of the recent advances in this newly recognized field. It should be noted that, while the concepts discussed extrapolate well to non-peptide strategies, much of the referenced work refers to peptides, reflecting the bias of the literature to date. And, while the mass screening of collections of diversity is a major topic unto itself (8), it is beyond the scope of this review to delve into the many screening technologies currently in use.

Molecular diversity may derive from chemical laboratory synthesis or from biological systems. Biologically generated diversity, well represented by the field of natural products, is ancient in its origins, and was well-established as a source of drugs during the last century of biomedical research. The range of diversity has extended in a major way beyond natural products only in the last decade. Progressive chemical and biological means for artificially generating diversity have now far outstripped the libraries of the past, with millions upon millions of compounds readily available through a variety of approaches. Recombinant systems such as bacteriophage excel in producing hundreds of millions of peptides incorporated into coat proteins. However, the building block set of 20 standard amino acids (19 L-amino acids plus glycine) extrapolates to a rather incomplete cross-section of the possible universe of diversity. In contrast, where chemical synthesis strategies may be less persuasive in terms of numbers, their diversity may be limitless because of the wider variety of building blocks possible in such an approach.

CHEMICAL DIVERSITY GENERATION

"Multi-Pin" Method - Around 1984, the field of chemically generated molecular diversity began when Geysen demonstrated that peptides could be synthesized in a reusable form in numbers orders of magnitude greater than before (9-11). In this method, peptides are synthesized on polyacrylate-grafted polyethylene rods arranged in a standard 96-well microtiter plate format. The format allows 96 individual peptides (or mixtures) to be synthesized simultaneously at the ends of the rods. The technique utilizes conventional solid phase peptide synthesis (SPPS) methods, readily available materials and instrumentation, and has been used, for example, to synthesize more than 3,000 peptides in a week. Moreover, peptides can be used in assays while still bound to the rods, allowing their re-use for 20 or more tests, or the peptides can be cleaved and then tested in solution-phase assays (12-14). One cleavage method yields products that contain a diketopiperazine moiety at the C-terminus, which seems not to affect biological activity in many cases (15). Through the use of new linkers or handles for attachment to the solid support, β-alanine acid or β-alanine methyl amide C-termini can also be generated (15). Alternatively, support-bound peptides can be cleaved with ammonia vapor to afford the corresponding C-terminal primary amides (16). An amino acid indexer that facilitates multi-pin use has been described (17).

The power of the multi-pin method in pharmaceutical research has been demonstrated recently in replacement net approaches to the determination of structure-activity relationships (SARs) in a series of potent hexapeptides that bind to endothelin receptors (18,19), and in studies of substance P (20,21). A general discussion of systematic screening for bioactive peptides has been published (22).

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The development of a method to prepare large numbers of peptides rapidly led naturally to strategies to map peptide epitopes. An epitope can be classified as continuous, that is, a linear sequence of amino acids homologous to a ligand, or to an antibody, receptor, or other binding molecule (a "ligate"). An epitope may also be discontinuous, that is, a pharmacophore consisting of non-adjacent amino acids brought into proximity by secondary or higher order structural features. Identification of continuous (i.e., sequential) epitopes using the multi-pin approach is accomplished by preparing a complete set of all possible overlapping synthetic peptides of given length homologous to the peptide sequence of interest. It is, of course, important that the length of the peptides is equal to or greater than that of the longest continuous epitope, usually in the range of 6-8 amino acid residues (23).

Discontinuous epitopes, probably more common than continuous epitopes, have been identified using the "mimotope" strategy (24-27). Making an entire mimotope library of a peptide sequence n-residues long, incorporating only the 20 standard amino acids, would require 20^n peptides, usually an unmanageable number (e.g., for an 8-mer, 20^n = 25,600,000,000 peptides). As an alternative, a stepwise approach can be taken to identify an optimum dipeptide sequence first, flanked by sequences of randomly incorporated amino acids. Once an optimum dipeptide sequence has been identified, remaining amino acids can be optimized sequentially (27). This combinatorial approach permits the rapid synthesis and biological evaluation of millions of individual peptides. The screening of such heterogeneous libraries, along with iterative selection and synthesis, allows the systematic identification of optimal peptide ligands. In this way, high affinity peptides for a monoclonal antibody known to recognize a discontinuous epitope have been identified from a library of 8-mers (24). As another example, the multi-pin method has proved useful in determining the effect of end groups, that is, amino terminal acylation, carboxy terminal amidation, or diketopiperazine termination, on the stimulatory activity of T-cell determinant peptides (28). A recent discussion considers practical strategies for the synthesis and testing of large libraries, taking into account the number of monomers available, as well as various optimization methods (29). The combination of epitope mapping with other SAR methods, made possible by the multi-pin or alternative MPS methods, has been used to enhance tumor necrosis factor (TNF) activity (30). It has also been suggested that peptide ligands, especially as selected from a vast library of random peptide sequences, may have potential in generating ligands for large-scale affinity chromatography (31).

Tea-Bag Approach - Among the first alternatives to multi-pin peptide synthesis was the tea-bag approach (32), where small amounts of resins representing individual peptides are enclosed in porous polypropylene containers, which resemble tea bags. Resin-containing tea bags are immersed in individual solutions of amino acids, and standard SPPS procedures are utilized. Deprotection and washing steps are accomplished by mixing the tea-bags together in a reaction vessel, followed by their subsequent separation for the next coupling. The peptides are cleaved in a multi-vessel HF apparatus (33,34). This method affords fully-characterizable, solution-phase peptides, which may yield more relevant data than solid-support bound peptides. Neither analysis nor separation is required for the identification of biologically-active peptides, since the biological assay identifies the tea-bags that contain active peptides. As with many of the other methods described herein, the specificity of antigen-antibody interactions can be readily determined at the level of individual amino acids (32). A semi-automated process allows the simultaneous synthesis of up to 120 different peptides (35). The method has been extended to a combinatorial approach (36), which permits the rapid synthesis and evaluation of millions of individual, unmodified, free peptides, in quantities useful for screening in solution. For example, from libraries of more than one million oligopeptides, nanomolar potency opioid ligands were identified using radioreceptor assays, and the antigenic determinant of a peptide recognized by a monoclonal antibody (MAb 125-10F3) was determined using a competitive enzyme-linked immunosorbent assay (ELISA) (37,38).

"Split Synthesis" Method - In another variation of MPS, combinatorial libraries of peptides are synthesized on resin beads using a split synthesis method, where a single peptide is attached to each bead (39-43). The split synthesis method has also been described as an "analog mixture-screening strategy", as reported for a study of neuropeptide Y and its receptor antagonists (44), and as "portioning-mixing" (45). In this approach, a polymeric support is divided into equal portions for coupling to each individual amino acid, and later recombined to create mixtures. Uniform coupling is ensured, since competition between amino acids due to unequal coupling rates is eliminated. Individual oligomers (or polymers) are combined during washing and deprotection, and then separated into individual portions for the next coupling. Resulting peptides exhibit a purely statistical

distribution of sequences. Through this combinatorial approach, a complete library of peptides can be prepared rapidly in approximately equimolar amounts. The library can be incubated with a ligate attached to a reporter molecule, bound acceptors being identified by visual inspection, and then binders may be physically removed with a micromanipulator. The peptide-on-bead is separated from the ligate, and then identified by microsequencing techniques. The approach may be extended to incorporate cleavable linkers, thus allowing solution-phase binding assays as well.

This technique has been applied to diverse mapping problems by various groups. A 512-component 6-mer library containing unnatural amino acids has been generated, using automated resin-splitting techniques, based on a sequence obtained from screening a diverse bacteriophage library with an anti-gp120 monoclonal antibody (46). Screening was accomplished using affinity selection methods. Importantly, the chemical and biological diversity approaches converged on similar, and in some cases identical, sequences. Pentamers and larger peptides that bind to anti-β-endorphin monoclonal antibodies have been studied using the split-resin approach (47). Defined peptide mixtures have also been used to identify human immunodeficiency virus (HIV) protease inhibitors (48). In the latter case, a transition-state analogue, statine, was included as a monomer at one position in a series of tetrapeptides, resulting in protease inhibitors with nanomolar potency.

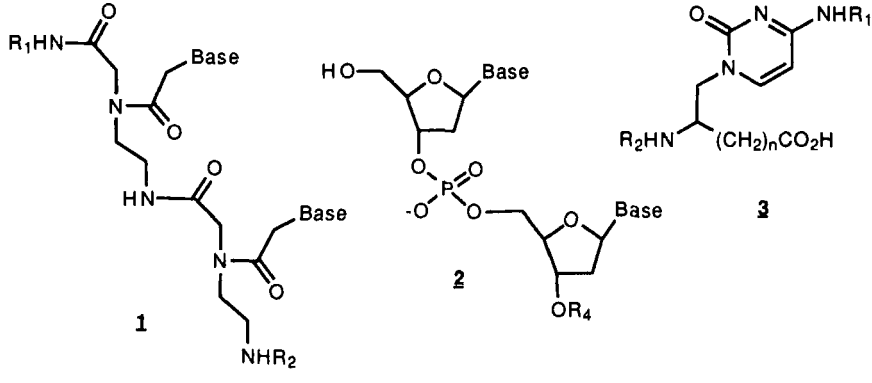
Chemical Synthesis on a "Chip" - The development of very large scale immobilized polymer synthesis (VLSIPS) resulted from advances in microelectronics, combining solid-phase synthesis with photolithography and miniaturization (49,50). In this technique, the use of photochemically labile protecting groups allows the light-directed, spatially addressable, parallel, chemical synthesis of thousands of oligomers at defined sites on a micromolar scale. Combinatorial masking strategies are used to generate large numbers of compounds in a small number of chemical steps. This technique allows the use of unnatural amino acids, nucleotides, and, in principle, any set of chemically compatible building blocks. The VLSIPS technology has been extensively reviewed in this series (2).

Other Synthetic Strategies and Solid Supports - Synthesis, cleavage, and purification of several peptides, in a single batch, simultaneously, has been automated (51). An iterative strategy to analyze the reactivity of peptide mixtures with antibodies employs a "bogus coin" strategy, wherein one tries to discern which member of a group of coins has a different weight than the others in a minimum number of weighings (52). Cellulose discs (53-56), stacked 100 or more per reaction column, have been used for the synthesis of individual peptides, oligonucleotides, and their libraries (57). By optimization of identical amino acid additions, the number of coupling cycles can be kept to a minimum. The rigid structure and low swelling properties of cellulose discs allow multiple couplings to be conducted under low pressure and continuous flow conditions. Along similar lines of research, rapid epitope analysis has been accomplished using cellulose paper sheets ("spot-synthesis") as absorptive membranes (58,59). Many compounds can be generated on one sheet, and both immobilized and solution peptides can be prepared. Cotton fabric also serves well as a solid support for SPPS and MPS, facilitating the synthesis of sequences that are difficult to prepare on polystyrene supports (60). Another variation utilizes two polymeric supports, Kel-F-g-styrene and polystyrene-1%-divinylbenzene, to synthesize simultaneously two related peptides, with most steps being carried out in the same reaction vessel under essentially identical conditions (61). Because the polymers have different densities, they can be separated by flotation at different stages of synthesis. The method works well for approximately 4-mers to 19-mers. Long-chain polystyrene-grafted polyethylene film can be handled as individually labeled and readily separable sheets, thus affording another solid support (62,63). Controlled-pore glass (CPG) resin has been used in SPPS to map B-cell determinants of pertussis toxin subunits and to raise and purify anti-peptide antibodies (64). The latter procedure is claimed to facilitate the handling of peptides of low solubility, and also provides an alternative to the usual protocol of peptide cleavage, purification, and conjugation to carrier protein.

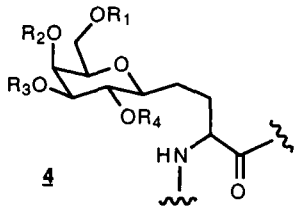
Automated Methods - A robotics-based, peptide or "peptoid" synthesizer, able to synthesize simultaneously up to 36 individual peptides or equimolar peptide mixtures (resin bound or solution phase), has been used to prepare 19 pools of 19 peptides each (41,65,66). This instrument automates the resin-splitting approach described earlier, as well as new processes for the preparation of oligo(N-substituted glycines) (NSGs) based on "submonomer" solid-phase synthesis (67). Moreover, automation has been extended with this robotics setup to deprotection and cleavage steps (68). The identification of potent ligands by affinity selection from equimolar peptide mixtures generated by robotic synthesis has been accomplished in a study of 10-mer epitopes that

bind an anti-HIV gp120 monoclonal antibody (69). A different automated instrument for multiple simultaneous peptide synthesis uses a flow-through apparatus with conventional solid supports contained in 48 individual reaction columns (70). Synthesis can also be performed on the surface of polyethylene pins. Performance of the instrument has been demonstrated through the synthesis of overlapping 14-mer peptides derived from the sequence of HIV reverse transcriptase (RT), as well as peptides related to TNF, the latter being useful in characterizing antibodies raised against intact protein. In addition, several MPS instruments based on traditional SPPS methods have been designed (58,71-76), and robotics systems for these synthesizers or for use with the multi-pin technique have been described (77). Resin-based peptide cyclization strategies using orthogonal protecting groups that may be accommodating to automation have been described (78). The automated synthesis of peptide C-terminal aldehydes, an important class of transition-state analogues and thus enzyme inhibitors, has been reported (79). In addition, a facile automated SPPS method for the synthesis of internally quenched, fluorogenic protease substrates has been described for use in the design of probes for Alzheimer's disease-associated proteases (80).

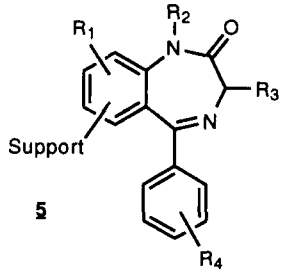
Non-Peptide Building Blocks - Amino acid building blocks, and thus peptides, represent much of the focus of molecular diversity generation to date, but several methods described above have also been used for the synthesis of multiple oligonucleotides. The use of oligonucleotide diversity in drug discovery and protein epitope targeting has been reviewed (81). An approach utilizing paper filter discs as the synthetic support for oligonucleotide synthesis has been described (82). The discs are stacked in different reaction vessels for the addition of either A, C, G, or T. After each coupling cycle, filter disks are reshuffled into appropriate vessels for the next step of chain elongation. In addition, silica-gel CPG, or teflon wafers containing CPG as the solid support, have been used to synthesize up to 100 oligonucleotides simultaneously (83). An asynchronous variation of the technique has been automated (84). Biological methods can also be used to prepare libraries of oligonucleotides, which are first selected for binding to an acceptor, and then amplified by polymerase chain reaction (PCR) (85,86). The selection of single-stranded DNA molecules ("aptamers") that bind and inhibit human thrombin is a noteworthy accomplishment in this respect (87). In these cases, however, the repertoire is limited not only by the requirement for nucleotides or nucleotide analogues that preserve specific Watson-Crick pairing, but also by the ability of the sequence to be copied by a polymerase. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide has been accomplished (88,89). With these polyamide nucleic acids (PNAs; **1**), the extraordinarily high stability of PNA-DNA hybrids demonstrates that the DNA backbone (**2**) can be effectively replaced by a polyamide. Molecular modeling studies have been reported on polyamide oligonucleotide analogues (90). The synthesis and oligomerization of diamino-oxo-pyrimidinealkanoic acids (**3**) as acyclic nucleic acid analogues has also been described (91). The multiple synthesis of carbohydrate containing compounds has also been explored. For example, 40 unique glycopeptides have been prepared manually using a multiple column peptide synthesizer (92). In terms of building blocks, stereoselective routes from glycals to asparagine-linked N-protected glycopeptides have been developed (93). Carbon-linked glycopeptides (**4**) may prove useful in extrapolating multiple compound synthesis to stable glycopeptide models (94).



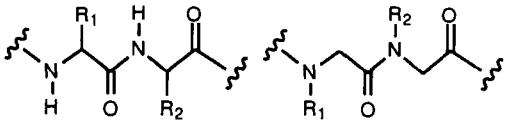
The convergence of solid-phase synthesis methods, a Nobel-prize winning contribution of the 1960s (95), with general building block strategies and pharmacophore-driven concepts such as peptoids (96), a significant contribution of the 1980s, is only now becoming commonplace. Yet the solid-phase synthesis of non-peptides is not a novel subject for organic chemists. For example, Dieckmann and other condensation reactions were explored in the 1970s and before (97,98). The preparation of monoacetates of symmetrical diols (99) and insect sex attractants (100,101) has also been accomplished using polymer-supported organic synthesis (102,103). However, the application of solid-phase non-peptide synthesis, or solid-phase compound synthesis (SPCS) to distinguish it from SPPS, to "rapid analoguing" in the pharmaceutical industry, is in a most seminal stage. Thus, for example, 1,4-benzodiazepines (**5**) have been prepared using solid-phase-based methods (104,105). Others have focused on peptide backbone (**6**) alternatives, including NSG (**7**) oligomers (106,107) and vinylogous polypeptide (**8**) oligomers (108). Still others have developed entirely new "scaffolds" to which potentially combinatorial functionalities can be attached, for example, 7-transmembrane G-protein coupled receptor binding derivatives (**9**) related to β-D-glucose (109), pyrrolinone-based peptidomimetics (**10**) possessing the conformation of a β-strand (110), and the design and synthesis of a peptidomimetic (**11**) with a steroidal backbone (111). A strategy to effect the rapid optimization of enzyme substrates using defined substrate mixtures has also been reported (112).



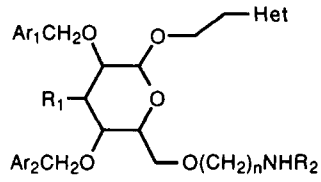
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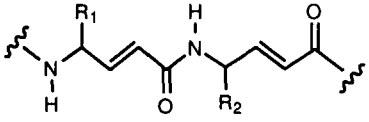
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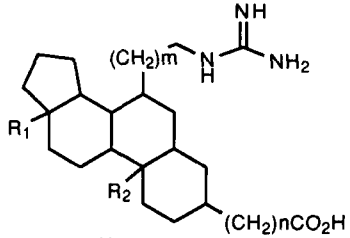
6 **7**



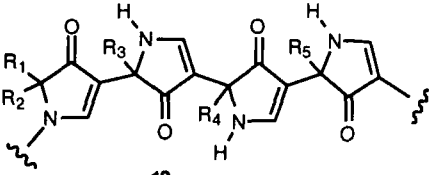
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BIOLOGICAL DIVERSITY GENERATION

Powerful biological methods have been described for the generation of peptide libraries displayed on the surface of a bacteriophage or phagemid (113,114). Such libraries contain tens to hundreds of millions of filamentous phage clones, each displaying a unique peptide sequence on the phage surface (and containing within the phage genome the DNA sequence encoding the peptide). A library can be prepared by incorporating randomized oligonucleotide sequences into the pIII gene, a phage-encoded gene for attachment to the host pilli. The nucleic acid sequence is thus manifested as a peptide sequence within the several identical gene III (pIII) proteins at one end of a virion. Alternatively, sequences can be cloned into the pVIII coat protein, several thousand copies of which appear on the phage body (115). Both pIII and pVII are synthesized with either their native or a compatible amino terminal signal sequence. These signal sequences, which are essential for translocation, are then cleaved as the polypeptide passes through the inner membrane of the envelope. In one approach, a ligate of interest is used to purify (by affinity) phage that display binding peptides, for example, allowing biotinylated ligate to react with a library, followed by "panning" the ligate/phage mixture on streptavidin-coated plates. Unbound phage are removed by washing, bound phage particles are released from ligate (e.g., using acid), and previously bound phage are propagated in *E. coli*. The amino acid sequences of binding peptides displayed by the phage are identified by sequencing the corresponding coding region of the viral DNA. Millions, approaching billions, of peptides can be surveyed rapidly for binding. Ideally, a range of peptide ligands are identified that recognize all available sites in and around a binding pocket.

Bacteriophage libraries display peptides with free amino termini. In some systems, where a free carboxylate is important for binding affinity, libraries displaying free carboxy termini may be more useful. As a result, methods have been developed to provide large libraries of peptides linked to the C-terminus of *lac* repressor, thus providing free carboxy termini (116). A consensus sequence related to dynorphin B has been obtained using this approach. In theory, other DNA binding proteins could be used in a similar manner to display peptide sequences. Some of the original work with phage peptide libraries incorporated hexapeptides. While epitopes for antibodies have been found by screening hexamer libraries, the success rate for finding high affinity ligands for receptors is markedly lower. As with chemically-generated libraries, a hexapeptide motif may not be suitable for identifying tight-binding peptides for many ligates, especially those that recognize folded determinants on proteins. Longer epitope regions and/or constrained epitopes may prove more useful in this respect. Libraries based on longer sequences, 15-mers, for example, have been developed (117,118). Although it is difficult to produce a complete library for a randomer of this size, 15-mers have the advantage of offering smaller determinants in the context of various flanking residues. In another phage library (119,120), the peptide epitope begins immediately after the signal peptide cleavage site, perhaps making this library more useful for finding peptide ligands since the diversity of phage bound peptides are not limited by a common amino terminal sequence.

There are multiple examples of the successful application of sophisticated phage libraries. Novel peptide antagonists for GPIIb/IIIa have recently been identified from a conformationally constrained cyclic disulfide phage library (121). Fusion of bovine pancreatic trypsin inhibitor (122) and a domain of growth hormone to pIII yields active proteins (123), demonstrating that complete protein domains can fold properly on the surface of phage. These methods allow the use of phage to clone and select genes by the binding properties of the expressed proteins, as well as enabling the rapid exploration of protein SARs. More recent work with phage libraries has identified peptide ligands for lectins (124,125). An efficient phage plaque screen has been developed for analysis of the interaction of mutagenized HIV-1 gp120 with human CD4 (126). Potent neutrophil elastase inhibitors displayed on M13 fusion phage have also been reported (127). A system for screening galactose-binding mutants has been described, using phage display of the ricin B chain and its single binding domains (128). Simplified methods for the construction, assessment, and rapid screening of phage peptide libraries have also been described (129).

Just as one might mimic the strategy of the immune system, driving the molecular evolution of non-immunological proteins on filamentous phage (130), one can apply phage technologies to antibodies. Thus, the generation of recombinant libraries encoding the entire murine repertoire of antibodies has been accomplished using PCR and *E. coli* expression techniques (131). Heavy and light chains were expressed in different vectors and combined artificially *in vitro* to afford monovalent Fab fragments. Random combinatorial libraries of rearranged heavy and light chains isolated by PCR are more easily screened using the phage system than by earlier methods because selection and

mutagenesis are facilitated, and also because antibodies to defined epitopes may be selected directly from phage libraries. Filamentous phage displaying functional antibody domains have been constructed. Single chain Fv domains, comprising H- and L-chain variable regions, have been prepared by linking the two regions with short, nondescript peptides, followed by phage expression (132). Alternatively, this has been accomplished *via* straightforward non-covalent heavy and light chain association (133,134), where one chain is attached to the phage protein embedded in the bacterial membrane, and the other is secreted into the periplasm where it can associate with the membrane-bound chain. Two-chain Fab domains have also been expressed wherein the H-chain comprising the variable region and the first subdomain of the constant region are fused to pVIII. The L-chain is again synthesized separately, and associates with the phage-attached H-chain (135). The phage-attached antibodies retain the antigen binding specificities of the parent antibodies. The design of phagemid vectors and *in vitro* maturation systems for antibody engineering have been reviewed (136). The selection of phage antibodies by binding affinity has been said to mimic affinity maturation (137). Semisynthetic combinatorial antibody libraries have also been reported (138).

Living organism-derived antibody libraries are intrinsically biased, thus some thought has been given to the concept of naive libraries for the generation of antibody combinatorial libraries. Random, chemically synthesized complementarity-determining regions (CDRs) could, in principle, be presented on a varied framework region. A step toward this goal has been taken by the synthesis of random heavy chains (CDR3), which significantly contribute to antigen-binding contacts and diversity. Thus, a library of Fab fragments differing only in HCDR3 has been displayed on phage and selected for antigen specificity (138). This semisynthetic approach to gene synthesis allows the production of human antibodies without immunization, and thus without the biases imposed in constructing libraries from animals. In an extension of these technologies, a system combining alkaline phosphatase, filamentous phage, and antibody Fab fragments has been developed, which allows one step ELISAs without immunization (139). Catalytic antibodies have been selected from combinatorial libraries (140). It has also been suggested that combinatorial antibody libraries might be used to clone the "fossil record" of an individual's immune response (141). An *in vitro* evolution procedure has been used to obtain RNA enzymes with a particular catalytic function (142).

The combination of chemical and biological diversity techniques has been contemplated for various purposes. For example, encoded combinatorial chemical libraries have been proposed as a means of identifying compounds bound to receptors (143,144). Extension of biological diversity, at present limited to natural building blocks such as the standard amino acids, awaits a more general and facile ability to conduct pseudo-biochemical mutagenesis using unnatural amino acids, for example, along the lines of recent work probing protein stability (145). Non-standard amino acids have been incorporated into peptides through expansion of the genetic code *via* ribosome-mediated translation (146). Unfortunately, the suppressor tRNA-based approach and other approaches are far from routine at this time.

Concluding Remarks - A dramatic convergence of technologies has turned molecular diversity generation and screening into a viable drug discovery tool. The power of multiple compound synthesis methodologies, whether chemical or biological, suggests that more compounds have been synthesized and screened in the 1990s than in the combined histories of the pharmaceutical and biotechnology industries pre-1990. Multiple compound synthesis has awakened a renewed interest in medicinal chemistry, compound libraries, and SARs that should carry throughout the 1990s and beyond and provide a valuable complement to more traditional discovery paradigms.

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SECTION VII. TRENDS AND PERSPECTIVES

Editor: James A. Bristol
Parke-Davis Pharmaceutical Research Division
Warner-Lambert Co., Ann Arbor, MI 48105

Chapter 34. To Market, To Market - 1992

June D. Strupczewski and Daniel B. Ellis
Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876

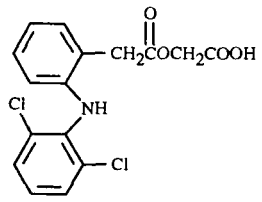
New chemical entities (NCEs), for human therapeutic use introduced for the first time during 1992 into the world marketplace totaled 36. This was the same number as introduced in 1991 (1), comparable to the 37 in 1990 (2) and slightly above the 33 NCEs introduced in 1989 (3).

Japan, after relinquishing the lead for one year to the United States, regained it in 1992 with 9 NCE introductions, 2 more than the United States, followed by Italy with 4 and France with 3. An additional 10 other countries were used to introduce the remaining 13 other NCEs. Approximately 60% of the new NCEs launched in 1992 originated in two countries; 13 in Japan and 8 in the United States while the United Kingdom had 5 with Spain, Germany and Italy 3 each. Antiinfectives, with two new quinolones, several cephalosporins and three AIDS-related products including a new antiviral, zalcitabine readily outnumbered all other therapeutic categories in 1992. Novel NCEs of special interest representing new classes of compounds include finasteride, the first agent for the treatment of benign prostatic hyperplasia; flosequinan for congestive heart failure and dexrazoxane, an adjuvant for reducing cardiac side effects associated with the anticancer anthracyclines. Other NCEs include the second proton pump inhibitor, lansoprazole and the third $5HT_3$ receptor antagonist, tropisetron, for the management of nausea induced by cancer chemotherapy; plus two platelet aggregation inhibitors, beraprost sodium and iloprost for the treatment of peripheral vascular disease.

In 1992, 26 NCE therapeutic agents were approved in the United States (4,5,6) slightly below the record number of 30 in 1991 but well above the 19 approved in 1990 (1,2). Included in these were eleven given the new "priority" classification by the FDA, of these, four were for AIDS-related conditions. Four of the 26 new approvals, atovaquone, desflurane, masoprocol and mivacurium chloride plus three approved in previous years, cefprozil, loracarbef and pentostatin, represented first worldwide introductions.

Aceclofenac (antiinflammatory) (7-9)

Country of Origin:	**Spain**
Originator:	**Prodes (Prodesfarma)**
First Introduction:	**Spain**
Introduced by:	**Prodes; Sanofi-Winthrop**
Trade Name:	**Airtal; Gerbin**
CAS Registry No.:	**89796-99-6**



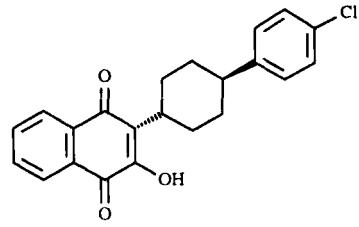
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Aceclofenac is a nonsteroidal antiinflammatory agent with analgesic and antipyretic properties. It is reported to be useful in the treatment of osteoarthritis, rheumatoid arthritis and pain associated with minor surgical procedures. Compared to ketoprofen in the treatment of rheumatoid arthritis, aceclofenac is substantially faster acting.

Atovaquone (antiparasitic) (10, 11)

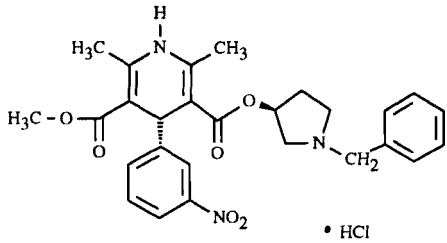
Country of Origin:	**United Kingdom**
Originator:	**Wellcome**
First Introduction:	**U.S.A.**
Introduced by:	**Wellcome**
Trade Name:	**Mepron**
CAS Registry No.:	**95233-18-4**



Atovaquone is an orally active antiprotozoal agent indicated for patients with mild to moderate AIDS-associated Pneumocystis carinii pneumonia who are intolerant to the first-line therapy of trimethoprim-sulfamethoxazole. It is also under investigation as a treatment for malaria and AIDS-associated toxoplasmosis.

Barnidipine Hydrochloride (antihypertensive) (12, 13)

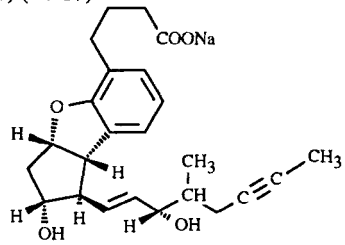
Country of Origin:	**Japan**
Originator:	**Yamanouchi**
First Introduction:	**Japan**
Introduced by:	**Yamanouchi**
Trade Name:	**Hypoca**
CAS Registry No.:	**104757-53-1**



Barnidipine hydrochloride is a vasodilating dihydropyridine calcium antagonist useful in the treatment of hypertension. In experimental animals, its hypotensive effect was more potent and longer acting than nicardipine, nifedipine and nitrendipine.

Beraprost Sodium (platelet aggregation inhibitor) (14-17)

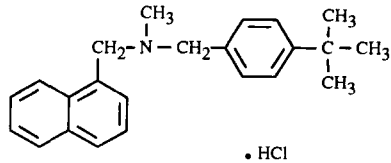
Country of Origin:	**Japan**
Originator:	**Toray**
First Introduction:	**Japan**
Introduced by:	**Toray; Yamanouchi; Kaken**
Trade Name:	**Dorner; Dorner; Procylin**
CAS Registry No.:	**88475-69-8**



Beraprost sodium is a new, orally active antithrombotic epoprostenol analog introduced as a treatment for peripheral vascular disease, including Raynaud's syndrome and Buerger's disease. The antiplatelet activity may be due to its elevation of cyclic AMP in platelets which is achieved by activating adenylate cyclase in the platelet membrane, followed by the inhibition of Ca^{++}- influx and thromboxane A2 formation.

Butenafine Hydrochloride (topical antifungal) (18, 19)

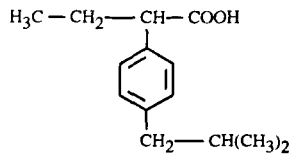
Country of Origin: **Japan**
Originator: **Kaken**
First Introduction: **Japan**
Introduced by: **Kaken; Hisamitsu**
Trade Name: **Mentax; Volley**
CAS Registry No.: **101827-46-7**



Butenafine hydrochloride is a new benzylamine antifungal which appears to block the squalene epoxidation step of sterol synthesis in fungi. Active against a broad spectrum of fungi, butenafine hydrochloride is more effective in a guinea-pig model of tinea pedis than clotrimazole, naftifine and tolnaftate. Its efficacy is partially attributed to its long retention time in the skin.

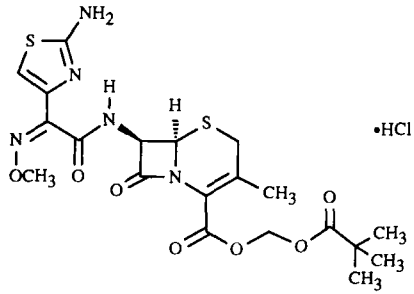
Butibufen (antiinflammatory) (20-22)

Country of Origin: **Spain**
Originator: **Juste**
First Introduction: **Argentina**
Introduced by: **E Merck**
Trade Name: **Butilopan**
CAS Registry No.: **55837-18-8**



Butibufen is a new phenylalkanoic antiinflammatory/analgesic useful in the treatment of rheumatologic conditions. In a study of patients with osteoarthritis, the onset of action of butibufen was faster with less frequent and milder side effects than indomethacin.

Cefetamet Pivoxil Hydrochloride (antibiotic) (23-25)

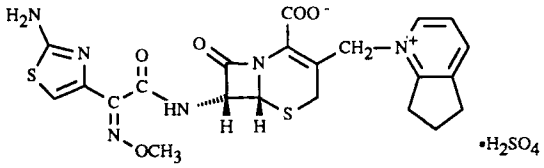


Country of Origin: **Japan**
Originator: **Takeda**
First Introduction: **Mexico**
Introduced by: **Roche**
Trade Name: **Globocef**
CAS Registry No.: **111696-23-2**

Cefetamet pivoxil hydrochloride, an ester prodrug of cefetamet, is an orally active cephalosporin introduced for the treatment of community-acquired respiratory and urinary tract infections. Roche indicated that this drug will possibly be the first third-generation cephalosporin to be launched in some countries. It is reportedly useful

in penicillin-resistant pneumococci, gonococci and H. influenzae infections, but ineffective against Staphylococci, P. aeruginosa and some non-fermenters.

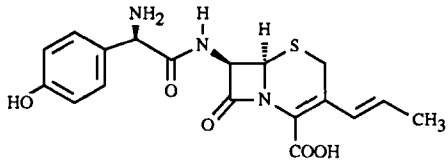
Cefpirome Sulfate (antibiotic) (26, 27)



Country of Origin:	**Germany**	Introduced by:	**Hoechst AG; Roussel Uclaf**
Originator:	**Hoechst AG**	Trade Name:	**Cefrom**
First Introduction:	**Sweden**	CAS Registry No.:	**98753-19-6**

Cefpirome sulfate is a new injectable cephalosporin indicated for the treatment of severe urinary and respiratory tract infections, including septicemia and nosocomial infections. Cefpirome sulfate is reported to be the first true fourth-generation cephalosporin due to its similar activities to third-generation antibiotics against Gram-positive bacteria and to first-generation antibiotics against Gram-negative bacteria. Its efficacy against P. aeruginosa is comparable to ceftazidime. The broad spectrum of activity includes Klebsiella, methicillin-sensitive staphylococci, penicillin-resistant pneumococci, E. coli which generates extended-spectrum beta-lactamases and multi-resistant organisms such as Citrobacter, Enterobacter and Serratia.

Cefprozil (antibiotic) (28-31)

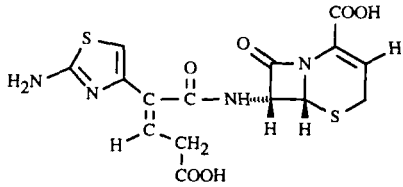


Country of Origin:	**U.S.A.**	Introduced by:	**Bristol-Myers Squibb**
Originator:	**Bristol-Myers Squibb**	Trade Name:	**Cefzil**
First Introduction:	**U.S.A.**	CAS Registry No.:	**92665-29-7**

Cefprozil is a new orally active cephalosporin useful in the treatment of otitis media, uncomplicated skin and skin structure infections plus upper/lower respiratory tract infections. Although structurally similar to cefadroxil, its spectrum of antibacterial activity is comparable to cefaclor, but exhibits greater activity against S. aureus, S. epidermidis, Listeria monocytogenes, Streptococci, H. influenzae, Propionibacterium acnes, Clostridium perfringens and difficile.

Ceftibuten (antibiotic) (32)

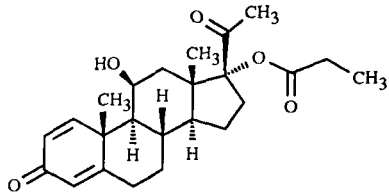
Country of Origin:	**Japan**
Originator:	**Shionogi**
First Introduction:	**Japan**
Introduced by:	**Shionogi**
Trade Name:	**Seftem**
CAS Registry No.:	**97519-39-6**



Ceftibuten is a new, once daily, orally active cephalosporin introduced as a treatment of Gram-negative bacteria-related urinary/respiratory tract and gynecological infections. *In vitro* studies of 359 strains of Gram-negative bacteria demonstrated that ceftibuten was superior to cefaclor and as active or slightly more active than cefixime and cefteram.

Deprodone Propionate (topical antiinflammatory) (33)

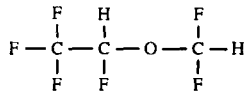
Country of Origin:	**United Kingdom**
Originator:	**Boots**
First Introduction:	**Japan**
Introduced by:	**SS Pharmaceutical**
Trade Name:	**Eclar**
CAS Registry No.:	**20424-00-4**



Deprodone propionate is a new topical antiinflammatory corticosteroid. In animal studies, it exhibited an extremely low skin irritation rate. Another potential use is as an antiasthmatic agent.

Desflurane (anesthetic) (34-36)

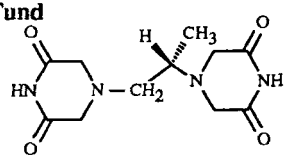
Country of Origin:	**U.S.A.**
Originator:	**Anaquest (BOC Healthcare)**
First Introduction:	**U.S.A.**
Introduced by:	**Anaquest**
Trade Name:	**Suprane**
CAS Registry No.:	**57041-67-5**



Desflurane is a new inhalation anesthetic introduced for induction and maintenance of general anesthesia in adults. Due to reports of respiratory irritation, desflurane may be used only for maintenance in children. Although almost structurally identical to isoflurane, a halogen replacement gives desflurane an improved pharmacokinetic profile. It is less soluble in blood and tissue and produces a fast onset of action and a more rapid recovery from anesthesia.

Dexrazoxane (cardioprotective) (37, 38)

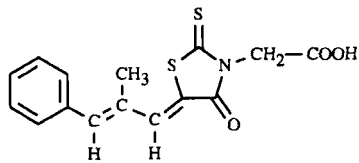
Country of Origin:	**United Kingdom**
Originator:	**Imperial Cancer Research Fund**
First Introduction:	**Italy**
Introduced by:	**EuroCetus**
Trade Name:	**Cardioxane**
CAS Registry No.:	**24584-09-6**



Dexrazoxane is the dextro-isomer of razoxane approved as a protectant against the cardiotoxicity of doxorubicin in breast cancer patients. This agent does not alter the anticancer effect of doxorubicin. Its mode of action suggests a chelation of free radicals. In mice, dexrazoxane also offers protection against cardiac side effects of epirubicin, but not mitoxantrone.

Epalrestat (antidiabetic) (39-42)

Country of Origin:	**Japan**
Originator:	**Ono**
First Introduction:	**Japan**
Introduced by:	**Ono**
Trade Name:	**Kinedak**
CAS Registry No.:	**82159-09-9**



Epalrestat is the second aldose reductase inhibitor to be introduced worldwide and the first to be launched in Japan. The compound is indicated for the treatment of diabetic neuropathy. It is also being investigated for diabetic retinopathy and nephropathy.

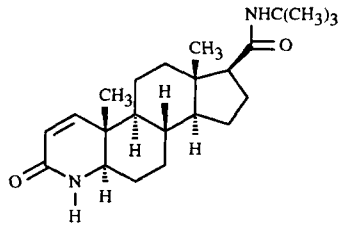
Factor VIII, Recombinant (hemostatic) (43, 44)

Country of Origin:	**U.S.A.**
Originator:	**Genetics Institute**
First Introduction:	**Sweden**
Introduced by:	**Baxter**
Trade Name:	**Recombinate**

Factor VIII, a recombinant DNA product, is useful for the management of bleeding episodes in patients with hemophilia A. A major advantage is that the synthetic version is free from HIV and hepatitis viruses. It should also prevent any shortages of this clotting factor.

Finasteride (5α-reductase inhibitor) (45-47)

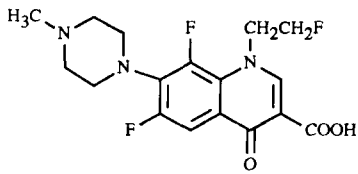
Country of Origin:	**U.S.A.**
Originator:	**Merck**
First Introduction:	**United Kingdom**
Introduced by:	**Merck**
Trade Name:	**Proscar**
CAS Registry No.:	**98319-26-7**



Finasteride, a novel 4-azasteroid, is a breakthrough in the treatment and control of benign prostatic hyperplasia. Mechanistically, it inhibits the prostatic-specific enzyme 5-alpha reductase, thereby decreasing the conversion of testosterone to dihydrotestosterone. It is reportedly effective in reducing urinary symptoms and prostatic volume and increasing maximal urinary flow rate. Finasteride is also being investigated as a treatment for prostatic cancer.

Fleroxacin (antibacterial) (48-50)

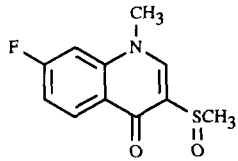
Country of Origin:	**Japan**
Originator:	**Kyorin**
First Introduction:	**Switzerland**
Introduced by:	**Roche**
Trade Name:	**Quinodis**
CAS Registry No.:	**79660-72-3**



Fleroxacin is a new, once daily trifluoroquinolone antibacterial structurally related to norfloxacin. Available in both oral and IV formulations, it is reportedly useful in the treatment of gonorrhea, bacterial enteritis, chronic bronchitis and urinary tract infections. Fleroxacin has a longer serum half-life (9-12 hours) than other drugs of this class, such as ciprofloxacin, enoxacin and norfloxacin.

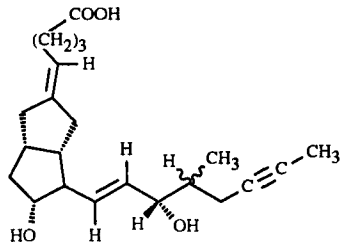
Flosequinan (cardiostimulant) (51-53)

Country of Origin:	**United Kingdom**
Originator:	**Boots**
First Introduction:	**United Kingdom**
Introduced by:	**Boots**
Trade Name:	**Manoplax**
CAS Registry No.:	**76568-02-0**



Flosequinan is a new mixed arteriolar and venous vasodilator useful for the treatment of patients with congestive heart failure with or without ACE inhibitors, but especially in those patients who are intolerant to ACE inhibitors. It does not cause reflex stimulation of the renin-angiotensin or sympathetic nervous system. How flosequinan achieves its direct vasodilating effect is not clear, it is thought to interfere with protein kinase C and the production of inositol triphosphate. Flosequinan is well absorbed orally, and with the relatively long half-life of its sulfone metabolite it is suitable for once a day administration.

Iloprost (platelet aggregation inhibitor) (54-57)



Country of Origin:	**Germany**	Introduced by:	**Schering AG**
Originator:	**Schering AG**	Trade Name:	**Ilomedin**
First Introduction:	**Austria; New Zealand; Switzerland**	CAS Registry No.:	**78919-13-8**

Iloprost is a stable epoprostenol derivative which acts as an inhibitor of platelet aggregation and is recommended for the treatment of peripheral vascular diseases. The compound was shown to be a potent agonist at PGE_2-sensitive and prostacyclin-sensitive receptors in animal studies. Iloprost is administered by IV infusion and has been reported to have beneficial effects in Buerger's and Raynaud's diseases as well as peripheral artery occlusive disease.

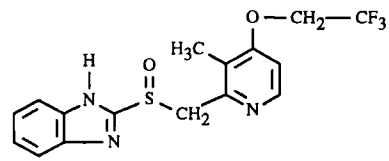
Interferon Gamma-1α, Recombinant (antineoplastic) (58, 59)

Country of Origin:	**Japan**
Originator:	**Suntory**
First Introduction:	**Japan**
Introduced by:	**Maruho**
Trade Name:	**Biogamma**

Interferon gamma -1α, a recombinant DNA product, has been introduced as a cytostatic agent for the treatment of cutaneous T-cell lymphoma. It is also being investigated for the treatment of ovarian cancer.

Lansoprazole (antiulcer) (60-63)

Country of Origin:	**Japan**
Originator:	**Takeda**
First Introduction:	**France**
Introduced by:	**Laboratoires Takeda; Houdé (Roussel Uclaf)**
Trade Name:	**Ogast; Lanzor**
CAS Registry No.:	**103577-45-3**

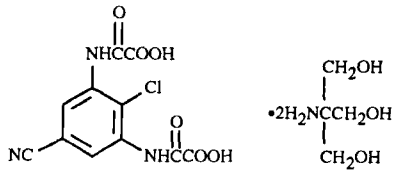


Lansoprazole is the second proton pump inhibitor approved as an antiulcer agent for the short term treatment of duodenal ulcer and gastroesophageal reflux disease. Like omeprazole, lansoprazole is reported to be more efficacious than the H_2-antagonists in

treating peptic ulcer disease. Other potential uses could be in the treatment of Zollinger-Ellison syndrome.

Lodoxamide Tromethamine (antiallergic ophthalmic) (64, 65)

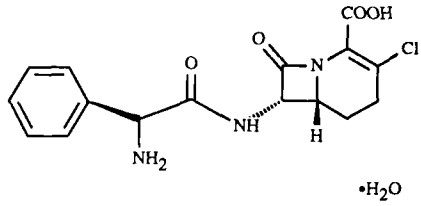
Country of Origin:	**U.S.A.**
Originator:	**Upjohn**
First Introduction:	**France**
Introduced by:	**Alcon**
Trade Name:	**Almide**
CAS Registry No.:	**63610-09-3**



Lodoxamide tromethamine was launched as an ophthalmic formulation for the treatment of allergic conjunctivitis. It appears to act as a mast cell stabilizer inhibiting degranulation and the release of histamine and other vasoactive products in a similar manner to sodium cromoglycate. The compound is also under investigation as an orally active antiallergic/antiasthmatic agent.

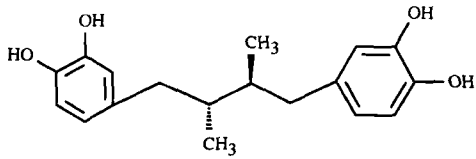
Loracarbef (antibiotic)(66-68)

Country of Origin:	**Japan**
Originator:	**Kyowa Hakko**
First Introduction:	**U.S.A.**
Introduced by:	**Lilly**
Trade Name:	**Lorabid**
CAS Registry No.:	**121961-22-6**



Loracarbef is a new orally active, broad-spectrum cephalosporin suitable for twice a day dosing. It is approved for use in both adults and children for the treatment of upper and lower respiratory tract infections, urinary tract and skin infections.

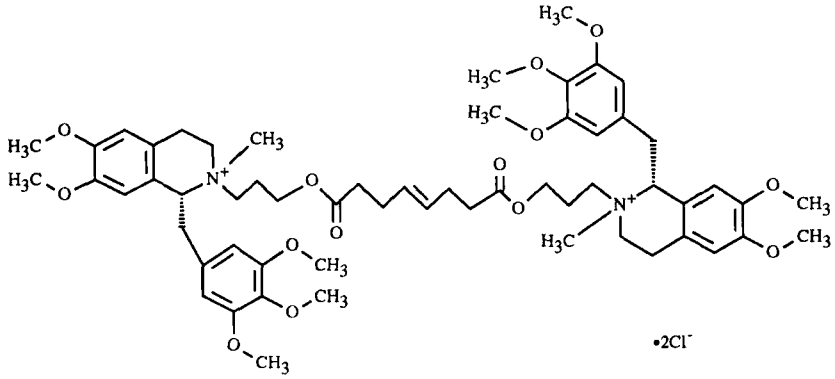
Masoprocol (topical antineoplastic) (69, 70)



Country of Origin:	**U.S.A.**	Introduced by:	**Block Drug**
Originator:	**Chemex**	Trade Name:	**Actinex**
First Introduction:	**U.S.A.**	CAS Registry No.:	**27686-84-6**

Masoprocol is a lipoxygenase inhibitor launched as a topical agent for the treatment of solar or actinic keratoses. It is the first new topical treatment for pre-malignant skin lesions introduced in twenty years. Masoprocol is obtained from the *Larrea tridentata* plant and is also under investigation for the treatment of lung, breast, colon and ovarian cancers.

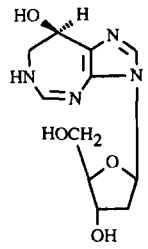
Mivacurium Chloride (muscle relaxant) (71-73)



Country of Origin:	**United Kingdom**	Introduced by:	**Wellcome**
Originator:	**Wellcome**	Trade Name:	**Mivacron**
First Introduction:	**U.S.A.**	CAS Registry No.:	**106861-44-3**

Mivacurium chloride, a mixture of three stereoisomers, is an intravenously administered, short-acting skeletal muscle relaxant introduced as an adjunct to general anesthesia. Structurally mivacurium chloride is closely related to doxacurium chloride introduced in 1991 by Wellcome as a muscle relaxant. It is a nondepolarizing neuromuscular blocking agent reportedly with a shorter duration of action and a more rapid rate of spontaneous recovery than other nondepolarizing agents. In extensive clinical trials mivacurium chloride was well tolerated with few side effects.

Pentostatin (antineoplastic) (74-76)

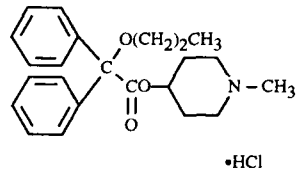


Country of Origin:	**U.S.A.**
Originator:	**Warner-Lambert (Parke-Davis)**
First Introduction:	**U.S.A.**
Introduced by:	**Warner-Lambert (Parke-Davis)**
Trade Name:	**Nipent**
CAS Registry No.:	**53910-25-1**

Pentostatin, an adenosine deaminase inhibitor, is an orphan drug introduced for the treatment of hairy cell leukemia. The agent is considered a significant advance over alpha-interferon, the only other drug available for this indication. The majority of patients treated with pentostatin have been reported to remain in remission for at least four years. It is also under investigation for the treatment of other kinds of leukemia and in transplant rejection.

Propiverine Hydrochloride (urologic) (77, 78)

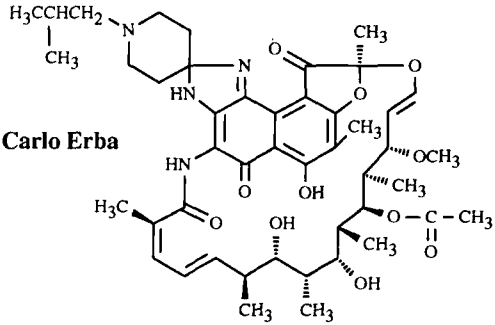
Country of Origin: **Germany**
Originator: **Apogepha**
First Introduction: **Germany**
Introduced by: **Apogepha**
Trade Name: **Mictonorm; Mictonetten**
CAS Registry No.: **54556-98-8**



Propiverine hydrochloride is a new urological agent developed for pollakuria and aconuresis. It appears to exhibit anticholinergic properties and in animal experiments inhibited KCl induced contractions in the bladder and ileum.

Rifabutin (antibacterial) (79-81)

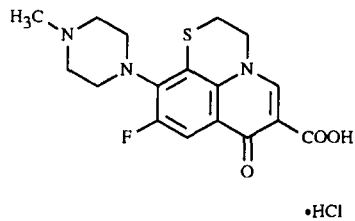
Country of Origin: **Italy**
Originator: **Archifar**
First Introduction: **Italy**
Introduced by: **Farmitalia Carlo Erba**
Trade Name: **Mycobutin**
CAS Registry No.: **72559-06-9**



Rifabutin, a rifamycin antibacterial derivative, is the first agent approved and introduced for the prevention of Mycobacterium avium complex (MAC) in AIDS patients. It is also indicated in combination chemotherapy for the prophylaxis and treatment of MAC infections in HIV positive patients and for newly diagnosed and chronic tuberculosis.

Rufloxacin Hydrochloride (antibacterial) (82, 83)

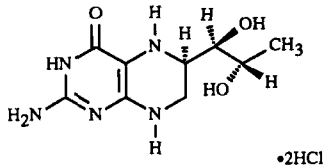
Country of Origin: **Italy**
Originator: **Mediolanum**
First Introduction: **Italy**
Introduced by: **Mediolanum; ISF (SmithKline Beecham)**
Trade Name: **Qari; Monos**
CAS Registry No.: **106017-08-7**



Rufloxacin hydrochloride is another fluoroquinolone orally active antibacterial structurally and mechanistically related to temafloxacin and tosufloxacin. It is useful for the treatment of respiratory and urinary tract infections and also chronic bacterial prostatitis. Good tissue penetration with long acting activity has been reported.

Sapropterin Hydrochloride (hyperphenylalaninemia) (84, 85)

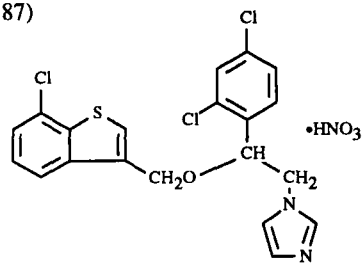
Country of Origin:	**Japan**
Originator:	**Suntory**
First Introduction:	**Japan**
Introduced by:	**Maruho**
Trade Name:	**Biopten**
CAS Registry No.:	**69056-38-8**



Sapropterin hydrochloride is a monoamine biosynthesis enhancer introduced for the treatment of hyperphenylalaninemia. Animal studies indicate that sapropterin hydrochloride increases brain levels of serotonin and enhances the release of acetylcholine from nerve terminals. The compound is also being investigated as a potential cognitive enhancer.

Sertaconazole Nitrate (topical antifungal) (86, 87)

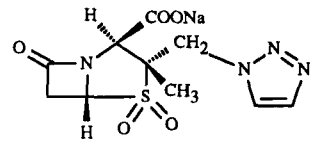
Country of Origin:	**Spain**
Originator:	**Ferrer**
First Introduction:	**Spain**
Introduced by:	**Ferrer; Laboratorios Robert (Ferrer)**
Trade Name:	**Dermofix; Zalain**
CAS Registry No.:	**99592-39-9**



Sertaconazole nitrate is a new broad spectrum imidazole antifungal agent introduced in a topical formulation for the treatment of cutaneous infections. Its mechanism of action is the inhibition of ergosterol synthesis and interference with the permeability of fungus cell membranes. An oral formulation is currently under investigation for the treatment of bucco-pharyngeal fungal infections.

Tazobactam Sodium (β-lactamase inhibitor) (88-90)

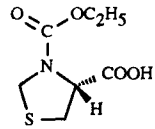
Country of Origin:	**Japan**
Originator:	**Taiho**
First Introduction:	**France**
Introduced by:	**Lederle (American Cyanamid)**
Trade Name:	**Tazocilline**
CAS Registry No.:	**89785-84-2**



Tazobactam sodium is a new triazolylmethyl beta-lactamase inhibitor launched in combination with the antibiotic piperacillin as tazocilline. Tazobactam sodium is active against penicillinases and wide spectrum of beta-lactamases. The combination product tazobactam/piperacillin is effective against a broad range of both Gram-positive and -negative organisms and is indicated for the treatment of lower respiratory tract, urinary tract, intra-abdominal, biliary and skin and soft tissue infections. It is expected to compete against the combination product augmentin (amoxicillin/clavulanate).

Telmesteine (mucolytic) (91, 92)

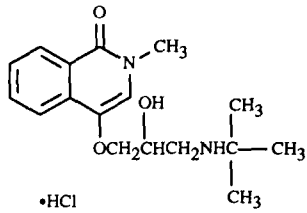
Country of Origin:	**Italy**
Originator:	**Yason (Medea)**
First Introduction:	**Italy**
Introduced by:	**Pulitzer; SPA**
Trade Name:	**Reolase; Muconorm**
CAS Registry No.:	**122946-43-4**



Telmesteine is a new mucolytic agent possessing expectorant and secretolytic activity introduced for the treatment of acute and chronic bronchitis. It is reported to have anti-elastase activity and to show effectiveness in pulmonary emphysema.

Tilisolol Hydrochloride (antihypertensive) (93, 94)

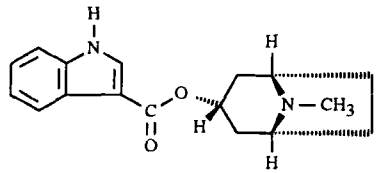
Country of Origin:	**Japan**
Originator:	**Nisshin Flour Milling**
First Introduction:	**Japan**
Introduced by:	**Maruho; Toyama**
Trade Name:	**Daim; Selecal**
CAS Registry No.:	**62774-96-3**



Tilisolol hydrochloride is a long acting vasodilating beta-blocker useful in the treatment of hypertension and angina. It is also reported to have antiarrhythmic properties. Due to its relatively long half-life, tilisolol hydrochloride is suitable for once a day dosing. It is currently being investigated as an antiglaucoma agent.

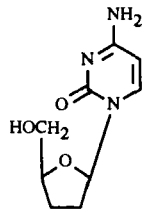
Tropisetron (antiemetic) (95-97)

Country of Origin:	**Switzerland**
Originator:	**Sandoz**
First Introduction:	**Netherlands**
Introduced by:	**Sandoz**
Trade Name:	**Novaban**
CAS Registry No.:	**89565-68-4**



Tropisetron is the third selective $5HT_3$-antagonist, after ondansetron and granisetron, to be introduced for the management of nausea and vomiting induced by cancer chemotherapy. Similar to the other two $5HT_3$-antagonists, tropisetron is superior to metoclopramide. The compound has also been reported to have antiarrhythmic activity.

Zalcitabine (antiviral) (98-100)



Country of Origin:	**U.S.A.**
Originator:	**National Cancer Institute (NIH)**
First Introduction:	**Austria**
Introduced by:	**Roche**
Trade Name:	**Hivid**
CAS Registry No.:	**7481-89-2**

Zalcitabine is an orally active dideoxynucleoside analog for combination use with zidovudine in advanced HIV infection and also as monotherapy for AIDS patients who cannot tolerate or have not responded to zidovudine. It has a similar mechanism of action (inhibition of reverse transcriptase) to didanosine which was introduced last year. Like didanosine, its side effect profile includes peripheral neuropathy. Unlike zidovudine, zalcitabine does not cause bone marrow suppression.

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Chapter 35. Medicinal Chemistry: Through a Glass Darkly

David J. Triggle,
State University of New York, Buffalo, New York

Introduction *O brave new world,*
That has such people in't!
William Shakespeare, The Tempest.

The patenting of a mouse predisposed to cancer and genetically engineered by Philip Leder and Timothy Stewart at Harvard University in April 1988 generated important scientific, commercial and ethical questions. Some five years later a self- imposed moratorium has ended and the United States Patent and Trademark Office is again issuing patents to a variety of genetically engineered animals, including those that exhibit prostate enlargement, develop insulin-resistant diabetes and produce human proteins including antibodies (1). There is little question but that these transgenic species will accelerate progress towards treatment of a variety of human disorders. Similarly, the production of transgenic plants, whilst generating less immediate ethical attention, will prove of value to the generation of medicinal products of natural origin such as scopolamine through the introduction of the hyoscyamine hydroxylase gene into Atropa belladonna (2).

During the same time period there has been a similar increase in human gene therapy (3). Some thirty-seven gene transfer trials have been approved worldwide and eighteen of these are designed to produce immediate therapeutic benefits. These include several trials inserting the gene for adenosine deaminase to alleviate the absence of a natural immune system and interleukin genes for several cancer types (4,5). With continued progress in overcoming the major problems of gene therapy including low gene transfer efficiency, poor cell targeting and premature gene turnoff it is clear that the horizons for human gene therapy will continue to expand.

The potential benefits of alleviating dopamine deficiency in the brains of Parkinson's patients by tissue implantation have been widely discussed from both clinical and ethical perspectives. Conventional dopamine replacement therapy suffers from well known and serious limitations. The most recent data indicate that fetal-tissue transplants into patients with idiopathic or MPTP- induced Parkinson's disease are clinically encouraging and may be highly beneficial for at least some patients (6,7).

Do these and similar developments in gene and tissue therapy signal the end of medicinal chemistry and the arrival at a solely biotechnology-oriented therapy mall where patients shop for both designer jeans and genes? Or, and more plausibly perhaps, will the molecular and medical sciences continue to adjust to a clinical interface that is positioned by science, economics and ethics?. It is clear that the issues of ethics raised by our rapid approach to the human genome will be of increasing importance to definition of any new clinical interface (8).

The History *heavenly messengers bringing evil reports of*
armies on the move and time running short
and famines and earthquakes and train wrecks
and the tearing down of the wall
bob dylan

The search for power and the explanation of natural events led naturally to the attribution of specific properties to plants and to their extracts. These properties were used in murder, magic and medicine, processes inextricably linked in man's history (9). Predictive, perhaps, of contemporary

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issues drugs were used also for their recreational properties; recent studies of Egyptian mummies have revealed the presence of cocaine, nicotine and hashish (10). Frequently, specific features of the plant were associated with and predictive of these properties. Thus the mandrake [*Mandragora officinarum*] was associated with the human form and special techniques were believed to be necessary in the collection of the plant to avoid madness or death. Those hominids partaking of Aminata muscaria would have been exposed to both the toxic and hallucinogenic effects of the muscarine, muscimol and ibotenic acid contained in this fungus. Aminata may have been the source of the soma of Vedic India and the inspiration for the soma of Aldous Huxley's Brave New World which had *"all of the advantages of Christianity and alcohol; none of their defects"*. One of the plagues of Egypt may well have been caused by the proliferation of dinoflagellates causing a red tide, fish killing and water poisoning (11).

The history of hashish dates back to at least 2000 BC and is associated with a considerable folk-lore. The word "assassin" derives from the Arabic for "hashish-eater" and the introduction of hashish to Europe by Marco Polo initiated investigations that have culminated in the identification of a specific neural receptor for the active constituent cannabinoids of marijuana and in the isolation of an endogenous ligand, anandamide, for this receptor (12).

The concept of specific receptors and sites of drug action has many origins. However, the work of Thomas Fraser and Alexander Crum Brown of Edinburgh University described in their publication of 1869, *"On The Connection Between Chemical Constitution and Physiological Action"*, provides both a definition of structure-activity relationships and a pioneering example of chemist-pharmacologist collaboration (13).

The definition of receptors as specific sites for drug action owes much to the work of John Newton Langley [1852-1925] and Paul Ehrlich [1854-1915]. Their work on the autonomic nervous system, toxins and chemo-therapeutic agents led to the concept of a receptor possessing both recognition and transduction components and of drugs possessing discrete regions subserving specific functions:

> *"It is convenient to have a term for the specially excitable constituent and I have called it the receptive substance. It receives the stimulus [recognition] and by transmitting it [transduction] causes contraction [response]"*
>
> J. N. Langley, 1906

> *"---they must possess a certain definite grouping which is chemically allied to one of the chemoreceptors of the parasite.---There must, therefore, be in addition to the fixing group another which brings about the destruction -- is to be characterized as the toxophoric"*
>
> P. Ehrlich, 1913

These predictions have been admirably fulfilled by the isolation and characterization of receptors themselves and by the assignment within receptors and individual molecules of domains responsible for such specific functions; these include ligand binding, voltage sensitivity and effector coupling in ion channels (**Figure 1**) and detection, initiation and delivery in the DNA cleaving enediynes (**Figure 2**) (14,15).

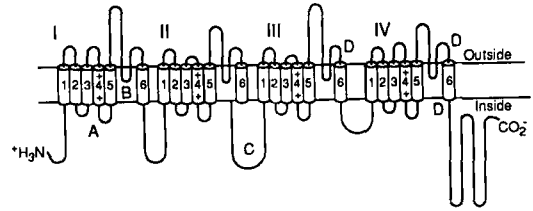


Figure 1. Domain organization of the alpha$_1$ subunit of the L-type voltage-gated Ca^{2+} channel. A, Voltage-sensor (S_4). B, Pore. C, E-C coupling. D, Drug binding sites. After Catterall (14).

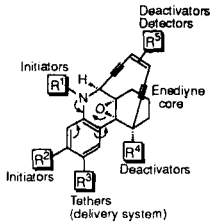


Figure 2. Domain organization of enediynes. After Nicolaou (l5).

Although he died during World War 1 Ehrlich was an optimist and writing some two years before his death:

> *"Now that the liability to, and danger of, disease are to a large extent circumscribed--the effects of chemotherapeutics are directed as far as possible to fill up the gaps left in this ring".*

Ehrlich had not, however, contemplated drug resistance, including the recent appearance of drug-resistant tuberculosis (16,17), nor presumably had he contemplated the dual impact of mass migrations and habitat destruction on the emergence of new and lethal infections including Lassa rift valley, Ross River, Crimean-Congo and HI viruses (18).

The Present *"The achievements of chemical synthesis are firmly bound to our attempt to break the shackles of disease and poverty"*

Roald Hoffmann, 1993.

The emergence of both old and new diseases focuses continuing efforts on drug discovery and the central role of medicinal chemistry. These foci are switching increasingly from the empirical and intuitive discovery processes to structure- and mechanism-based discovery routes. These changes are coupled to the emergence of new targets and roles for medicinal chemistry that interface increasingly with molecular biology.

New cellular messengers with unconventional properties continue to define new targets. Nitric oxide, or a labile derived species, is now established to mediate physiological and pathological events in cardiovascular, secretory and neuronal cells (19-21). The recent postulate that CO may also be a messenger species (22,23) serving, with nitric oxide, a common guanylyl cyclase effector defines new signalling pathways that link space and time to coordinate the activities and functions of cell populations (24). A major role for such species can be envisaged in the regulation of the brain and its multiple neuronal populations. The modulation of the activities of such novel species presents both major opportunities and problems of site specificity and delivery.

The critical role of calcium as a cellular regulator has been recognized for over a century (25). Cyclic adenosine diphosphate ribose [cADP-ribose] is the latest messenger serving to modulate the release of intracellular Ca^{2+} and perhaps representing the endogenous ligand for the ryanodine- sensitive Ca^{2+} release channel (26,27). Not only is this messenger an important link between nutrient levels and insulin release in pancreatic beta-cells, but it may serve quite generally to link nutrients with Ca^{2+} fluxes. The availability of both cADP ribose and inositol phosphate pathways to control intracellular Ca^{2+} release provides a parallel to the multiple entry processes for Ca^{2+} during cell excitation and provides an additional base for the spatial and temporal control of this messenger (28). Additionally, this discovery raises again the issue of endogenous ligands for other types of Ca^{2+} channels (29).

New targets demand new ligands and more subtle definition of the ligand-target interactions. Target definition by X-ray and NMR as structure-based drug design promises to become a predictive component of the discovery process (30). The design of two-fold symmetric inhibitors of HIV protease followed directly from the discovery that the retroviral enzymes are heterodimers (31-33). Other examples are likely to follow for other soluble proteins. Membrane-bound proteins constitute, however, particular problems for both X-ray and NMR methods, but the high resolution electron microscopic techniques applied to two-dimensional membrane sheets including bacteriorhodopsin and porin may well be useful for other systems (34,35). The determination of the structure of the human growth hormone complexed with the soluble extra-cellular domain of its receptor suggests an alternative strategy of membrane proeins (36). Additionally, extension to ligand interactions with other macromolecules including DNA as in the glucocorticoid-DNA complex is occurring (37).

Gene isolation has been a major force in the character-ization, expression and use in screening of pharmacological receptors. Two lessons learned are that pharmacological specificity may depend upon a single amino acid residue and that for receptors composed of subunits only a limited number of permutations may exist. The antagonist specificity of the rodent 5-HT_{1B} receptor is determined by a single residue [asparagine-355] from that of a human brain receptor [threonine-355] with overall sequence homology, although both receptors bind 5-HT equally well (38). The benzodiazepine- sensitive $GABA_A$ receptor, composed of multiple alpha, beta, gamma and delta subunits derived from some 15 genes and with several hundred thousand possible permutations, exists in very restricted distribution and combinations (39,40).

The generation of lead structures and their optimization constitutes a central theme of medicinal chemistry. This theme places continuing demands on synthetic chemistry including the specification of new catalytic processes and consideration of chiral interactions (41,42). Increasingly, these demands will interface with biological processes including catalytic antibodies for disfavored chemical reactions (43) and chiral control through enzymatic and biomimetic processes (41). The mimicking of the biological world by chemistry has become extremely productive. The award of the 1987 Nobel Prize in Chemistry to Cram, Lehn and Pedersen was an explicit acknowledgment of the role of molecular recognition processes and of the analogies between host-guest chemistry and ligand-receptor interactions (45,46). Thus, a simple A4B6 cyclooligomer of trimesic acid and R,R- diaminocyclohexane exhibits very high chirality and side-chain specificity in peptide recognition (47). In turn, chemistry is expanding the synthetic capabilities of biological systems. The "natural" biological code specifies only 20 amino acids. Expansion of this code is possible through the creation of new codon-anticodon pairs that specify novel residues (48). The non-standard pair (iso-C)AG:(iso-dG)CU specifies L-iodotyro-sine and indicates the possibilities of a semi-synthetic genetic code. A general method for incorporation of unnatural amino acids into proteins derives from replacement of a specific codon by the "blank" nonsense codon TAG through oligonucleotide-directed mutagenesis and use of a suppressor tRNA directed against this codon that has been aminoacylated by the amino acid residue of interest (49).

Although ligand design may ultimately be completely rational and predictable from target structures, iterative processes of design and selection will continue to be of major significance despite its traditional heavy consumption of time and money. It is of increasing importance to devise methods that facilitate lead generation and selection. A variety of combinatorial techniques are now available that generate libraries of compounds from amino acids or nucleotides. These range from extensions of peptide resin synthesis to the use of fusion phage which express on their surface a single peptide (50-54). Affinity-mediated selection followed by physical or sequence-based localization permits the characterization of a lead structure. A combination of these processes entitled "encoded combinatorial chemistry" has been proposed in a theoretical treatment by Brenner and Lerner to consist of parallel syntheses where each monomeric chemical unit is encoded by a nucleotide sequence (**Figure 3**) (55). Active molecules are affinity-selected and identity and amplification achieved through the PCR applied to the retrogenetic tag. In principle, this method is not restricted to amino acid or nucleotide precursors and any set of building blocks could be employable. Such molecular construction kits are now becoming available and some are termed, appropriately enough, "Molecular Meccano" (56-58). A final component to the process of ligand selection **may be** accomplished by closing the circle of variation/mutation, selection and amplification/ replication to provide evolution-directed selection (59-61).

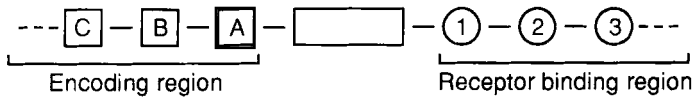


Figure 3. Encoded combinatorial synthesis. After Brenner and Lerner (55).

The Future *give me back my broken night*
my secret room my secret life
its lonely here
Leonard Cohen

Advances in synthetic methodologies notwithstanding, the future of medicinal chemistry will make increasing demands on chiral control, on peptidomimetics and on target- and site-specific delivery.

Exploration of specific cellular receptors as surrogate entry loci for peptides and proteins offers significant opportunity for target-specific delivery particularly if combined with mechanisms to selectively enhance the expression of delivery receptors. Antibodies to the transferrin receptors can cross the blood-brain barrier by internalization through these receptors localized in brain capillary endothelial cells. Antibodies conjugated to nerve growth factor deliver the latter to neurons and enhance the survival of cholinergic and noncholinergic neurons transplanted into the eye (62).

Similar techniques may be used to accomplish receptor- mediated gene delivery (63,64). Gene transfer technology is systems. Retroviral vectors may be particularly and selec-tively useful because of their ability to transfer genes only into dividing cells. Thus, murine fibroblasts expressing a retroviral vector containing the herpes simplex thymidine kinase gene transduced proliferating rat glioma cells. Addition of the anti- herpes drug ganciclovir regressed the gliomas since these cells only have the herpes enzyme (65).

The goal of peptide mimicry is the discovery of structural leads, or the development of strategies for molecular design, which result in bioavailable peptide analogs (66). The development of a strategy is awaited and most active compounds derive from lead identification and optimization. However, "retrofitting" of such leads as the benzodiazepine nucleus found in ligands for opiate, CCK, gastrin and glutamate receptors (76-71) and the 1,4-dihydropyridine structure as a component of drugs active at several voltage-gated ion channels (72) is likely to be productive.

The importance of chirality to the drug-receptor interaction has been recognized since at least the work of Cushney (73). An increasing need to consider chirality as a theme in all aspects of drug discovery from synthesis and manufacture to pharmacodynamics, pharmacokinetics and toxicology derives from the new FDA guidelines (74-77). Indeed, since 1985 almost 50% of the optically active molecules developed as drugs have been developed as active isomers and there are probably some 50-60 currently marketed racemates that are prime candidates for chiral switching (76,78). Fortunately, perhaps, for the pharmaceutical industry molecules such as palytoxin with 64 dissymmetric carbon atoms and 10^{21} isomers are rare (79). However, even simple chiral molecules may present complex pharmacology because of opposing chiral effects of pharmacokinetic and pharmacodynamic factors. This may be particularly so for drugs active at ion channels where state-dependent interactions may modify both quantitatively and qualitatively the expression of drug action (80,81). Thus, the stereoselectivity of local anesthetic interactions at Na^+ channels varies according to the frequency of stimulus and the activation mode (82,83).

At the synthetic level we may expect increased emphasis on enantioselective catalysis using metal complex catalysts as a key component of the manufacturing process (84). For bio-catalysts there will unquestionably continue to be increasing interest in the "custom synthesis" of enzymes engineered for specific functions and conditions. The first example of the "ultimate" enzyme has been reported with the synthesis of the all-D form of HIV-1 protease (85-87). This enzyme exhibits a chiral specificity opposite to that of the naturally occurring L form and it may

be generally predicted that enantiomeric proteins will exhibit reciprocal chiral specificity in all aspects of their interactions. These reciprocal chiral speci-ficities of D-and L- proteins are of considerable interest; they suggest that in worlds alternative to ours their FDA will be the mirror image of ours. ***On the other hand,*** .....

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aceclofenac	antiinflammatory	1992	28, 325
acetohydroxamic acid	hypoammonuric	1983	19, 313
acipimox	hypolipidemic	1985	21, 323
acitretin	antipsoriatic	1989	25, 309
acrivastine	antihistamine	1988	24, 295
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
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
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
amrinone	cardiotonic	1983	19, 314
amsacrine	antineoplastic	1987	23, 327
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
APSAC	thrombolytic	1987	23, 326
arbekacin	antibiotic	1990	26, 298
argatroban	antithromobotic	1990	26, 299
arotinolol HCl	antihypertensive	1986	22, 316
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
atovaquone	antiparasitic	1992	28, 326
auranofin	chrysotherapeutic	1983	19, 314
azelaic acid	antiacne	1989	25, 310
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
bambuterol	bronchodilator	1990	26, 299
barnidipine hydrochloride	antihypertensive	1992	28, 326
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benazepril hydrochloride	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
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beraprost sodium	platelet aggreg. inhibitor	1992	28, 326
betaxolol HCl	antihypertensive	1983	19, 315
bevantolol HCl	antihypertensive	1987	23, 328

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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
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budralazine	antihypertensive	1983	19, 315
bunazosin HCl	antihypertensive	1985	21, 324
bupropion HCl	antidepressant	1989	25, 310
buserelin acetate	hormone	1984	20, 316
buspirone HCl	anxiolytic	1985	21, 324
butenafine hydrochloride	topical antifungal	1992	28, 327
butibufen	antiinflammatory	1992	28, 327
butoconazole	topical antifungal	1986	22, 318
butoctamide	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	hypertensive	1988	24, 298
calcipotriol	antipsoriatic	1991	27, 323
camostat mesylate	antineoplastic	1985	21, 325
carboplatin	antibiotic	1986	22, 318
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
cefbuperazone sodium	antibiotic	1985	21, 325
cefdinir	antibiotic	1991	27, 323
cefetamet pivoxil hydrochloride	antibiotic	1992	28, 327
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
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cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefotetan disodium	antibiotic	1984	20, 317
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cefpiramide sodium	antibiotic	1985	21, 325
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cefteram pivoxil	antibiotic	1987	23, 330
ceftibuten	antibiotic	1992	28, 329
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celiprolol HCl	antihypertensive	1983	19, 317
centchroman	antiestrogen	1991	27, 324
centoxin	immunomodulator	1991	27, 325
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
choline alfoscerate	nootropic	1990	26, 300

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cicletanine	antihypertensive	1988	24, 299
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
cilazapril	antihypertensive	1990	26, 301
cinitapride	gastroprokinetic	1990	26, 301
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
citalopram	antidepressant	1989	25, 311
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
dapiprazole HCl	antiglaucoma	1987	23, 332
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
delapril	antihypertensive	1989	25, 311
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
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
disodium pamidronate	calcium regulator	1989	25, 312
divistyramine	hypocholesterolemic	1984	20, 317
dopexamine	cardiostimulant	1989	25, 312
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
ebastine	antihistamine	1990	26 302
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
epirubicin HCl	antineoplastic	1984	20, 318
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eptazocine HBr	analgesic	1987	23, 334

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
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 VIII	hemostatic	1992	28, 330
famotidine	antiulcer	1985	21, 327
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenticonazole nitrate	antifungal	1987	23, 334
filgrastim	immunostimulant	1991	27, 327
finasteride	5α-reductase inhibitor	1992	28, 331
fisalamine	intestinal antiinflammatory	1984	20, 318
fleroxacin	antibacterial	1992	28, 331
flomoxef sodium	antibiotic	1988	24, 302
flosequinan	cardiostimulant	1992	28, 331
fluconazole	antifungal	1988	24, 303
fludarabine phosphate	antineoplastic	1991	27, 327
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
fluticasone propionate	antiinflammatory	1990	26, 303
flutoprazepam	anxiolytic	1986	22, 320
flutropium bromide	antitussive	1988	24, 303
fluvoxamine maleate	antidepressant	1983	19, 319
formoterol fumarate	bronchodilator	1986	22, 321
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosinopril sodium	antihypertensive	1991	27, 328
fotemustine	antineoplastic	1989	25, 313
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303
gemeprost	abortifacient	1983	19, 319
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
goserelin	hormone	1987	23, 336
granisetron hydrochloride	antiemetic	1991	27, 329
guanadrel sulfate	antihypertensive	1983	19, 319
halobetasol propionate	topical antiinflammatory	1991	27, 329
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
idarubicin hydrochloride	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
iloprost	platelet aggreg. inhibitor	1992	28, 332
imipenem/cilastatin	antibiotic	1985	21, 328

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indobufen	antithrombotic	1984	20, 319
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
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
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
lamotrigine	anticonvulsant	1990	26, 304
lansoprazole	antiulcer	1992	28, 332
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
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
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
loracarbef	antibiotic	1992	28, 333
loratadine	antihistamine	1988	24, 306
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
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
mifepristone	abortifacient	1988	24, 306
milrinone	cardiostimulant	1989	25, 316
miokamycin	antibiotic	1985	21, 329
misoprostol	antiulcer	1985	21, 329

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
mivacurium chloride	muscle relaxant	1992	28, 334
mitoxantrone HCl	antineoplastic	1984	20, 321
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
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
nabumetone	antiinflammatory	1985	21, 330
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306
naftifine HCl	antifungal	1984	20, 321
naltrexone HCl	narcotic antagonist	1984	20, 322
nedocromil sodium	antiallergic	1986	22, 324
nemonapride	neuroleptic	1991	27, 331
nicorandil	coronary vasodilator	1984	20, 322
nilutamide	antineoplastic	1987	23, 338
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
nipradilol	antihypertensive	1988	24, 307
nisoldipine	antihypertensive	1990	26, 306
nitrefazole	alcohol deterrent	1983	19, 322
nitrendipine	hypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
omeprazole	antiulcer	1988	24, 308
ondansetron hydrochloride	antiemetic	1990	26, 306
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
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
paroxetine	antidepressant	1991	27, 331
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pemirolast potassium	antiasthmatic	1991	27, 331
pentostatin	antineoplastic	1992	28, 334
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide hydrochloride	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pinacidil	antihypertensive	1987	23, 340
pirarubicin	antineoplastic	1988	24, 309

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
piroxicam cinnamate	antiinflammatory	1988	24, 309
plaunotol	antiulcer	1987	23, 340
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propentofylline propionate	cerebral vasodilator	1988	24, 310
propiverine hydrochloride	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
quazepam	hypnotic	1985	21, 332
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
ramipril	antihypertensive	1989	25, 317
ranimustine	antineoplastic	1987	23, 341
rebamipide	antiulcer	1990	26, 308
remoxipride hydrochloride	antipsychotic	1990	26, 308
repirinast	antiallergic	1987	23, 341
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
rimantadine HCl	antiviral	1987	23, 342
rokitamycin	antibiotic	1986	22, 325
romurtide	immunostimulant	1991	27, 332
ronafibrate	hypolipidemic	1986	22, 326
rosaprostol	antiulcer	1985	21, 332
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin hydrochloride	antibacterial	1992	28, 335
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin hydrochloride	hyperphenylalaninemia	1992	28, 336
sargramostim	immunostimulant	1991	27, 332
schizophyllan	immunostimulant	1985	22, 326
sertaconazole nitrate	topical antifungal	1992	28, 336
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
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatropin	hormone	1987	23, 343
spizofurone	antiulcer	1987	23, 343
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	B-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344

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tazobactam sodium	ß-lactamase inhibitor	1992	28, 336
tazanolast	antiallergic	1990	26, 309
teicoplanin	antibacterial	1988	24, 311
telmesteine	mucolytic	1992	28, 337
temafloxacin hydrochloride	antibacterial	1991	27, 334
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
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
tilisolol hydrochloride	antihypertensive	1992	28, 337
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
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
toremifene	antineoplastic	1989	25, 319
tosufloxacin tosylate	antibacterial	1990	26, 310
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
tropisetron	antiemetic	1992	28, 337
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
xamoterol fumarate	cardiotonic	1988	24, 312
zalcitabine	antiviral	1992	28, 338
zidovudine	antiviral	1987	23, 345
zolpidem hemitartrate	hypnotic	1988	24, 313
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

GENERIC NAME	INDICATION	YEAR INTRODUCED	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19, 319
mifepristone		1988	24, 306
nitrefazole	ALCOHOL DETERRENT	1983	19, 322
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
propacetamol HCl		1986	22, 325
sufentanil		1983	19, 323
suprofen		1983	19, 324
desflurane	ANESTHETIC	1992	28, 329
propofol		1986	22, 325
sevoflurane		1990	26, 309
azelaic acid	ANTIACNE	1989	25, 310
nedocromil sodium	ANTIALLERGIC	1986	22, 324
repirinast		1987	23, 341
tazanolast		1990	26, 309
lodoxamide tromethamine	ANTIALLERGIC OPHTHALMIC	1992	28, 333
gallopamil HCl	ANTIANGINAL	1983	19, 319
cibenzoline	ANTIARRHYTHMIC	1985	21, 325
encainide HCl		1987	23, 333
esmolol HCl		1987	23, 334
moricizine hydrochloride		1990	26, 305
pilsicainide hydrochloride		1991	27, 332
tiracizine hydrochloride		1990	26, 310
amlexanox	ANTIASTHMATIC	1987	23, 327
ibudilast		1989	25, 313
pemirolast potassium		1991	27, 331
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

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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
carboplatin		1986	22, 318
carumonam		1988	24, 298
cefbuperazone sodium		1985	21, 325
cefdinir		1991	27, 323
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
erythromycin acistrate		1988	24, 301
flomoxef sodium		1988	24, 302
imipenem/cilastatin		1985	21, 328
isepamicin		1988	24, 305
lenampicillin HCl		1987	23, 336
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
sultamycillin tosylate		1987	23, 343
temocillin disodium		1984	20, 323
mupirocin	ANTIBIOTIC, TOPICAL	1985	21, 330
chenodiol	ANTICHOLELITHOGENIC	1983	19, 317

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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
moclobemide		1990	26, 305
paroxetine		1991	27, 331
setiptiline		1989	25, 318
sertraline hydrochloride		1990	26, 309
tianeptine sodium		1983	19, 324
toloxatone		1984	20, 324
acarbose	ANTIDIABETIC	1990	26, 297
epalrestat		1992	28, 330
tolrestat		1989	25, 319
granisetron hydrochloride	ANTIEMETIC	1991	27, 329
ondansetron hydrochloride		1990	26, 306
tropisetron		1992	28, 337
centchroman	ANTIESTROGEN	1991	27, 324
fenticonazole nitrate	ANTIFUNGAL	1987	23, 334
fluconazole		1988	24, 303
itraconazole		1988	24, 305
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
sertaconazole nitrate		1992	28, 336
sulconizole nitrate		1985	21, 332
apraclonidine HCl	ANTIGLAUCOMA	1988	24, 297
befunolol HCl		1983	19, 315
dapiprazole HCl		1987	23, 332
levobunolol HCl		1985	21, 328
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
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
carvedilol		1991	27, 323
celiprolol HCl		1983	19, 317
cicletanine		1988	24, 299
cilazapril		1990	26, 301
delapril		1989	25, 311
dilevalol		1989	25, 311
doxazosin mesylate		1988	24, 300
enalapril maleate		1984	20, 317
enalaprilat		1987	23, 332
felodipine		1988	24, 302
fosinopril sodium		1991	27, 328
guanadrel sulfate		1983	19, 319
isradipine		1989	25, 315
ketanserin		1985	21, 328
lacidipine		1991	27, 330
lisinopril		1987	23, 337
manidipine hydrochloride		1990	26, 304
moxonidine		1991	27, 330
nilvadipine		1989	25, 316
nipradilol		1988	24, 307
nisoldipine		1990	26, 306
perindopril		1988	24, 309
pinacidil		1987	23, 340
quinapril		1989	25, 317
ramipril		1989	25, 317
rilmenidine		1988	24, 310
terazosin HCl		1984	20, 323
tertatolol HCl		1987	23, 344
tiamenidine HCl		1988	24, 311
tilisolol hydrochloride		1992	28, 337
trimazosin HCl		1985	21, 333
aceclofenac	ANTIINFLAMMATORY	1992	28, 325
AF-2259		1987	23, 325
amfenac sodium		1986	22, 315
butibufen		1992	28, 327

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deflazacort		1986	22, 319
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
tenoxicam		1987	23, 344
fisalamine	ANTIINFLAMMATORY, INTESTINAL	1984	20, 318
osalazine sodium		1986	22, 324
alclometasone dipropionate	ANTIINFLAMMATORY, TOPICAL	1985	21, 323
aminoprofen		1990	26, 298
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
sumatriptan succinate		1991	27, 333
dronabinol	ANTINAUSEANT	1986	22, 319
amsacrine	ANTINEOPLASTIC	1987	23, 327
bisantrene hydrochloride		1990	26, 300
camostat mesylate		1985	21, 325
doxifluridine		1987	23, 332
enocitabine		1983	19, 318
epirubicin HCl		1984	20, 318
fludarabine phosphate		1991	27, 327
flutamide		1983	19, 318
fotemustine		1989	25, 313
idarubicin hydrochloride		1990	26, 303

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interferon gamma-1α		1992	28, 332
interleukin-2		1989	25, 314
lonidamine		1987	23, 337
mitoxantrone HCl		1984	20, 321
nilutamide		1987	23, 338
pentostatin		1992	28, 334
pirarubicin		1988	24, 309
ranimustine		1987	23, 341
toremifene		1989	25, 319
vinorelbine		1989	25, 320
masoprocol	ANTINEOPLASTIC, TOPICAL	1992	28, 333
atovaquone	ANTIPARASITIC	1992	28, 326
ivermectin		1987	23, 336
droxidopa	ANTIPARKINSONIAN	1989	25, 312
pergolide mesylate		1988	24, 308
lidamidine HCl	ANTIPERISTALTIC	1984	20, 320
gestrinone	ANTIPROGESTOGEN	1986	22, 321
acitretin	ANTIPSORIATIC	1989	25, 309
calcipotriol		1991	27, 323
amisulpride	ANTIPSYCHOTIC	1986	22, 316
remoxipride hydrochloride		1990	26, 308
zuclopenthixol acetate		1987	23, 345
diacerein	ANTIRHEUMATIC	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

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benexate HCl	ANTIULCER	1987	23, 328
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
plaunotol		1987	23, 340
rebamipide		1990	26, 308
rosaprostol		1985	21, 332
roxatidine acetate HCl		1986	22, 326
roxithromycin		1987	23, 342
sofalcone		1984	20, 323
spizofurone		1987	23, 343
teprenone		1984	20, 323
troxipide		1986	22, 327
didanosine	ANTIVIRAL	1991	27, 326
foscarnet sodium		1989	25, 313
ganciclovir		1988	24, 303
rimantadine HCl		1987	23, 342
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
flumazenil	BENZODIAZEPINE ANTAG.	1987	23, 335
bambuterol	BRONCHODILATOR	1990	26, 299
doxofylline		1985	21, 327
formoterol fumarate		1986	22, 321
mabuterol HCl		1986	22, 323
oxitropium bromide		1983	19, 323
salmeterol hydroxynaphthoate		1990	26, 308
APD	CALCIUM REGULATOR	1987	23, 326
clodronate disodium		1986	22, 319
disodium pamidronate		1989	25, 312
gallium nitrate		1991	27, 328
ipriflavone		1989	25, 314
dexrazoxane	CARDIOPROTECTIVE	1992	28, 330
bucladesine sodium	CARDIOSTIMULANT	1984	20, 316
denopamine		1988	24, 300
dopexamine		1989	25, 312
enoximone		1988	24, 301

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flosequinan		1992	28, 331
ibopamine HCl		1984	20, 319
milrinone		1989	25, 316
vesnarinone		1990	26, 310
amrinone	CARDIOTONIC	1983	19, 314
xamoterol fumarate		1988	24, 312
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
nicorandil	CORONARY VASODILATOR	1984	20, 322
azosemide	DIURETIC	1986	22, 316
muzolimine		1983	19, 321
alglucerase	ENZYME	1991	27, 321
cinitapride	GASTROPROKINETIC	1990	26, 301
cisapride		1988	24, 299
erythropoietin	HEMATOPOETIC	1988	24, 301
factor VIII	HEMOSTATIC	1992	28, 330
malotilate	HEPATOPROTECTIVE	1985	21, 329
buserelin acetate	HORMONE	1984	20, 316
goserelin		1987	23, 336
leuprolide acetate		1984	20, 319
nafarelin acetate		1990	26, 306
somatropin		1987	23, 343
sapropterin hydrochloride	HYPERPHENYLALANINEMIA	1992	28, 336
cadralazine	HYPERTENSIVE	1988	24, 298
nitrendipine		1985	21, 331
binfonazole	HYPNOTIC	1983	19, 315
brotizolam		1983	19, 315
butoctamide		1984	20, 316
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

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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
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
bucillamine	IMMUNOMODULATOR	1987	23, 329
centoxin		1991	27, 325
thymopentin		1985	21, 333
filgrastim	IMMUNOSTIMULANT	1991	27, 327
interferon gamma-1b		1991	27, 329
lentinan		1986	22, 322
pegademase bovine		1990	26, 307
romurtide		1991	27, 332
sargramostim		1991	27, 332
schizophyllan		1985	22, 326
ubenimex		1987	23, 345
cyclosporine	IMMUNOSUPPRESSANT	1983	19, 317
mizoribine		1984	20, 321
muromonab-CD3		1986	22, 323
sulbactam sodium	ß-LACTAMASE INHIBITOR	1986	22, 326
tazobactam sodium		1992	28, 336
telmesteine	MUCOLYTIC	1992	28, 337
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
timiperone		1984	20, 323
bifemelane HCl	NOOTROPIC	1987	23, 329
choline alfoscerate		1990	26, 300
exifone		1988	24, 302

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idebenone		1986	22, 321
indeloxazine HCl		1988	24, 304
levacecarnine HCl		1986	22, 322
nizofenzone fumarate		1988	24, 307
oxiracetam		1987	23, 339
beraprost sodium	PLATELET AGGREG.	1992	28, 326
epoprostenol sodium	INHIBITOR	1983	19, 318
iloprost		1992	28, 332
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
finasteride	5α-REDUCTASE INHIBITOR	1992	28, 331
surfactant TA	RESPIRATORY SURFACTANT	1987	23, 344
APSAC	THROMBOLYTIC	1987	23, 326
alteplase		1987	23, 326
tiopronin	UROLITHIASIS	1989	25, 318
propiverine hydrochloride	UROLOGIC	1992	28, 335
clobenoside	VASOPROTECTIVE	1988	24, 300
cadexomer iodine	WOUND HEALING AGENT	1983	19, 316
epidermal growth factor		1987	23, 333