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PREFACE

A continuing objective of *Annual Reports in Medicinal Chemistry* has been to combine regular updates of significant areas of research in medicinal chemistry with emerging areas of biological science, destined to impact the future discovery and development of new therapeutic agents.

Volume 26 retains the familiar format of previous volumes, this year with 32 chapters. Sections I - IV are related to specific medicinal agents, with annual updates on antipsychotics, anti-allergy agents, and antibacterials, where the objective is to provide the reader with the most important new results in a particular field. This year, for the first time, the chapter on antihypertensives has been replaced with three more mechanistically-related chapters: renin angiotensin system, potassium channel activators, and vasoactive peptides. Also included are chapter topics accorded frequent recent updates in previous volumes, including antidepressants, serotonin, and antivirals. Several chapters cover topics of high current interest, including neuronal calcium channels, neurokinin antagonists, thrombosis, CCK, osteoporosis, and mechanism-based immunosuppressants. The increased focus on viral disease therapy today is reflected in three chapters on this topic, with an appropriate emphasis on AIDS. The importance of molecular biology as an enabling technology to medicinal chemical research is apparent in all of the chapters in these sections.

Sections V and VI continue to emphasize important emerging areas of medicinal chemistry and biology as well as the interface between these disciplines. Chapters in Section V, Topics in Biology, include cytokine receptors, amyloidogenesis, bacterial adhesions, and the ras GTPase cycle.

Chapters in Section VI continue to be directed to newer technologies, this year with a focus on molecular diversity which is a topic of high current interest as a means of expediting drug discovery. Also included are chapters on distance geometry methodology and antisense nucleotides as therapeutics.

Trends and Perspectives (Section VII) contains the traditional chapter on NCE introductions and concludes with a look toward the future on perspectives in human gene therapy.

Over the past year, it has been my pleasure and good fortune to work with 6 highly professional section editors and 64 authors, whose excellent and incisive contributions constitute Volume 26.

James A. Bristol
Ann Arbor, Michigan
May, 1991

SECTION I. CNS AGENTS

Editor: John M. McCall, The Upjohn Company
Kalamazoo, MI 49001

Chapter 1. New Anxiolytics.

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Berlex Laboratories, 110 East Hanover Avenue, Cedar Knolls, NJ 07927

Introduction - In the past, Annual Reports in Medicinal Chemistry has reviewed the topics of anxiolytics, anticonvulsants and sedative/hypnotics in the same chapter. This was largely due to the broad and rather non-specific activities of the benzodiazepines. While the disease state of anxiety is not yet fully understood, the structure elucidation of the macromolecular complex consisting of chloride channel, barbiturate receptor, GABA receptor and benzodiazepine (BZ) receptor has increased the likelihood that the specificity of future therapeutics can be improved. Although not simplifying the challenge, the existence of $GABA_A$ and $GABA_B$ receptors and the subtyping of the BZ receptor into ω_1, ω_2 and ω_3 subtypes via selective ligands and molecular biology has further raised hopes that a much improved anxiolytic can yet be discovered. In addition, accumulating evidence has continued to support the original electrophysiological findings that first suggested that cholecystokinin (CCK) may have a role in inducing anxiety. Furthermore, the reported beneficial effects of 5-HT_1, 5-HT_2 and 5-HT_3 receptor subtype ligands in anxiety have kept alive interest in serotonin (5-HT) as a modulator of anxiety.

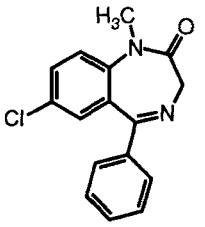
These and other developments help to maintain a high level of research interest in an area where, compared to other central nervous system diseases, existing therapy is relatively safe and effective. However, anxiety disorders can have serious consequences. Suicide attempts have been reported among 20% of people with chronic panic disorders and 12% of those who experience panic attacks (1). Panic disorders are currently treated with monoamine oxidase inhibitors, benzodiazepines and tricyclic antidepressants. Clomipramine, a tricyclic, has recently been approved by the FDA for obsessive-compulsive disorder. The serotonin uptake inhibitor's, chemistry, clinical pharmacology, pharmacokinetics and adverse effects have been described in detail. Its typical tricyclic side-effects may be its major disadvantage (2).

MOLECULAR BIOLOGY OF ANXIETY

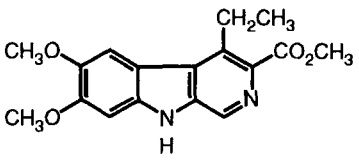
A detailed review of the molecular biology of $GABA_A$ receptors has summarized the significant regional variation and distribution of receptor subtypes. Different $GABA_A$ receptor (3) subtypes exhibit differing sensitivity to benzodiazepines, GABA and other physiological regulators and environmental stimuli. The exact repertoire of receptor subtypes necessary for fully active $GABA_A$ receptors is still elusive. However, progress has been made on establishing the structural requirements of fully functional $GABA_A$ receptors in mammalian brain (4). The γ_2-subunit cDNA of rat brain has been cloned and coexpressed with the α_1 and β_1 subunits in Xenopus oocytes. The GABA-induced currents of the recombinant receptor were enhanced by diazepam (1) and inhibited by DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate

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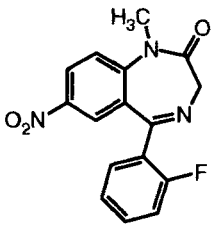
2.) In situ hybridization was employed to probe in which neuronal population the subunits might be present (4). Further in situ hybridization histochemistry studies revealed the mRNA for the α_1-, β_1- and γ_2-subunits to be co-localized in the mitral cells of the olefactory bulb, pyramidal cells of the hippocampus and granule cells of dentate gyrus and cerebellum. These studies suggest extensive receptor heterogeneity (5). Similarly, it has been shown that flunitrazepam (3) and DMCM do not appear to act at isosteric sites. Differences in the responsiveness of $GABA_A$ receptors to these compounds is attributed to different subunit compositions in cultured cells. Thus, the agonist flunitrazepam potentiates GABA responses in γ_2 subunit-containing receptors, whereas DMCM reduces the GABA-induced change in α_5 subunit-containing receptors (6).



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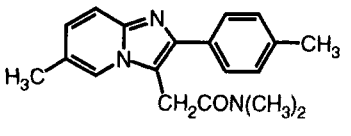
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The cDNA clones for the complete coding sequence of diazepam binding inhibitor (DBI) have been isolated from rat, human and bovine expression libraries. Southern blot analysis using the DBI cDNA suggests that human and rat DBI may be encoded by a multiple family of 4-6 genes (7).

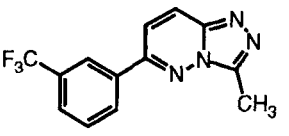
THE BENZODIAZEPINE RECEPTOR

Benzodiazepine Receptor Subtypes - It has been proposed that agonist differential selectivity for the benzodiazepine, ω_1, ω_2 and ω_3 receptor subtypes may account for the varied pharmacological action of the known anxiolytics which owe their activity to this mechanism of action. This classification and widely accepted proposal has stimulated renewed interest in the discovery of novel anxiolytics in which the pharmacological profile is fine-tuned by the compounds' affinity at the receptor subtypes.

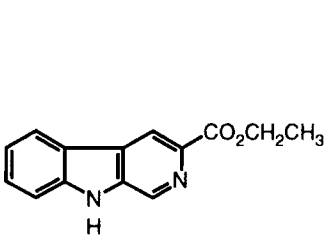
The designations ω_1, ω_2 and ω_3 receptor subtypes are replacing the use of BZ_1, BZ_2 and BZp (8). Receptors of the ω_1-subtype are identified by zolpidem (4), partial agonist CL-218872 (5), inverse agonist β-CCE (ethyl-β-carboline-3-carboxylate, 6) and antagonist CGS 8216 (7). The ω_2-subtype is non-selectively recognized by benzodiazepines and by the antagonist flumazenil (8). The peripheral BZp designation is the ω_3 receptor subtype recognized by PK 11195 (9) and Ro 5-4864 (10).



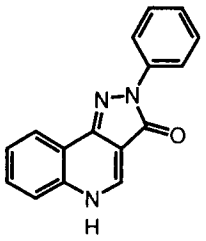
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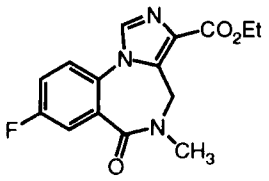
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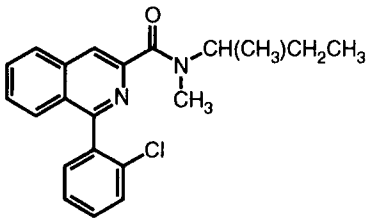
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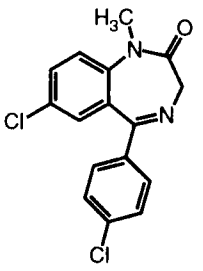
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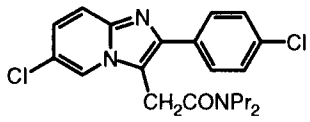


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The concept has been developed and is now widely held that the side-effect profile exhibited by the benzodiazepines is a result of their lack of subtype selectivity (9). Consequently, it is believed that an agent with selectivity for one or a specific profile of activity at all three will result in a superior if not ideal anxiolytic drug. Profiling of alpidem (11) has shown that while it is a potent ligand at the ω_1 and ω_3 receptor subtypes, it has low affinity for ω_2. This is in contrast to other pharmacologically related compounds (Table 1) (9).



11

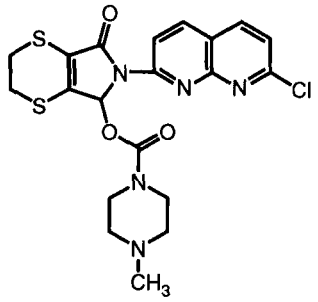
Table 1. Relative Binding Affinities of Selected Benzodiazepine Receptor Ligands

	ω_1	ω_2	ω_3
alpidem	7	303	1
zolpidem	26	180	1036
CL-218872	390	1500	>10,000
flunitrazepam	2.5	1.4	234

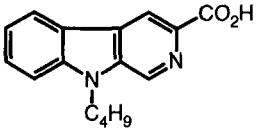
The imidazo[1,2-a]pyridine, alpidem, binds to the ω_1 and ω_3 receptor subtypes from human brain with Kd of 1.67 nM and 0.3 nM respectively. The binding of [^{3}H]-alpidem in rat cerebral cortex is enhanced by GABA, but unaffected by chloride ions and pentobarbital. The latter observation differentiates it from the BZ class of compounds. Its high affinity and high selectivity for subtypes ω_1 and ω_3 may explain its selective anxiolytic activity. Although it is the most potent ligand at ω_3 receptor subtype known, and since currently prescribed benzodiazepines are weak ligands at ω_3, the relevance of ω_3 activity to the anxiolytic effects of alpidem remains unclear and open to speculation. In another study, the type I BZ receptor agonists, zolpidem and alpidem have been compared to diazepam in their ability to induce spontaneous electrical activity of substantia nigra pars reticulata (SNR) neurons. It appears that zolpidem, a hypnotic, has a more potent inhibitory effect in this assay than either of the anxiolytics, alpidem and diazepam (10). These effects were judged to be benzodiazepine receptor mediated since they were blocked by flumazenil.

The ability of the fragments of diazepam binding inhibitor (DBI) to displace [^{3}H]-flumazenil and [^{3}H]-Ro 5-4864 from their receptor subtypes has been investigated (11). Triakontatetraneuropeptide (TTN) is the C-terminal containing portion of DBI possessing amino acid residues 17-50. Since residues 17-50, 22-50, 26-50 and 33-50 all interacted with the benzodiazepine sites while α-amidated DBI 33-50 and DBI 19-41 did not, it was concluded that the carboxy terminal fragment is essential for interaction. Furthermore, the differing propensities of these fragments to form α-helices were implicated in receptor recognition and subtype discrimination.

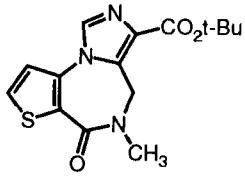
An in vivo PET study has shown that suriclone (**12**) is able to rapidly displace [^{11}C]-flumazenil from baboon cortex in a dose dependent manner and at pharmacologically active doses (12).



12



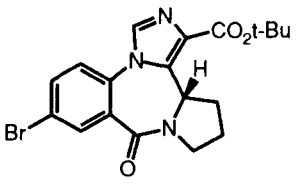
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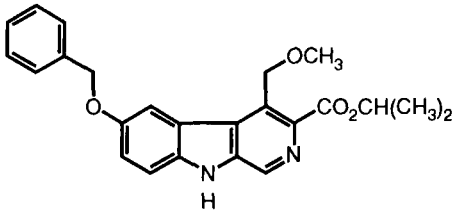
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Benzodiazepine Full, Partial and Inverse Agonists - Ligands, which induce less than a full functional response at the BZ receptor, are partial agonists that may be novel anxiolytics (13). It is speculated that a partial agonist may be associated with fewer sedative, muscle relaxant, physical dependence and tolerance side-effects. Flumazenil, a central BZ receptor antagonist, and n-butyl-β-carboline-3-carboxylate (BCCB, **13**), an inverse agonist, have been used to support the existence of a mechanism mediated through BZ agonists, which inhibits the acquisition of stress-related behavior (14). Evidence has been presented to confirm that the partial inverse-agonist, Ro 19-4603 (**14**), is anxiogenic and to support that this is an intrinsic property of inverse agonists at the BZ receptor (15). Bretazenil (**15**), a partial agonist, produces little sedation at doses significantly higher than anticonvulsant doses (16). Physical

dependence liability appears to be lower as evidenced by no effect doses (40 mg/kg) of bretazenil in primates (17). The β-carboline, abecarnil (ZK 112119, **16**) is a partial agonist at central benzodiazepine receptors with lower intrinsic activity than bretazenil. It is reported to be inactive in serotonin, α-adrenergic, opiate and dopamine receptor subtype binding assays (18). In contrast, it displaces [^{3}H]-lormetazepam from rat cerebral cortical and cerebellar membranes with IC_{50} values of 0.82 nM and 0.73 nM, respectively. It is an effective anxiolytic in animal models with little or no effect in muscle relaxation or motor coordination, and is relatively weak in potentiating the effects of ethanol and barbiturates. Its anticonvulsant effect in rodents and baboons has also been described in detail (19). It is orally available and under clinical evaluation (20).

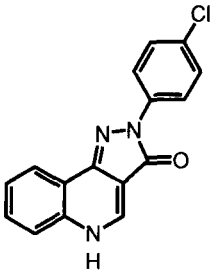


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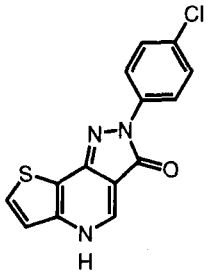


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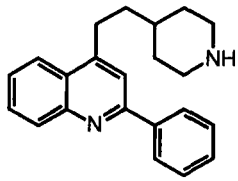
Other Benzodiazepine Ligands - The clinical utility and potential for structural variation driven by classical medicinal chemistry has made the BZs an area which has received much attention with the goal of discovering anxioselective compounds (21) without side-effects. A series of pyrazolo[4,3-*c*]pyridines (22) related to partial agonist CGS 9896 (**17**) (23) has been published. A comparison has been made with the thienopyridine series (24). Certain members of these series may differ from CGS 9896 and diazepam in their being equally potent in the rat Vogel test but significantly less active in the anti-pentylenetetrazole test, e.g., **18**. This is a profile (25) reminiscent of pipequaline (PK 8165, **19**).



17



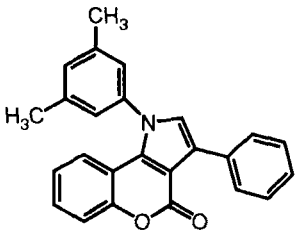
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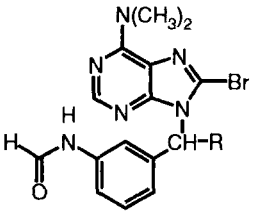
19

In another series of potent BZ receptor ligands, exemplified by **20** , the GABA shift or GABA ratio was used to distinguish between agonists, inverse agonists, and antagonists (26). A publication on a series of 9-benzylpurines report BZ receptor binding activity similar to diazepam and no significant activity in the Geller-Seifter conflict schedule (27). In the presence of GABA, compound **21** showed an unaltered IC_{50} value of 11 nM, hence suggesting antagonist activity. In contrast, a subsequent report by the same group has shown that addition of an α-methyl group to give the 9-(1-phenylmethyl)purines, e.g. **22**, resulted in a loss of BZ receptor affinity. This loss of affinity was attributed to steric interactions between the 8-bromo and 9-(1-phenylethyl) substituents which result in a conformation that is not optimal for interaction with the BZ receptor (28).

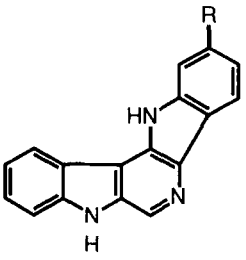
A detailed computer-assisted analysis of the BZ receptor inverse agonist site (29) and a series of dihydropyridodiindoles which are rigid planar ligands for the receptor (30) have been published. The most potent members of the series bear a substituent in the 2-position and possess nanomolar activity in displacing [^{3}H]-diazepam from rat cortical membranes. The methoxy analogue (**23**) was the most potent partial inverse agonist of the series in vivo in mice as measured by the proconvulsant activity. Interestingly, the chloro derivative (**24**) exhibited no proconvulsant activity, but blocked the anticonvulsant effect of diazepam.



20



21 R = H
22 R = CH_3

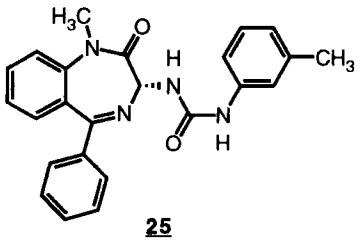


23 R = OCH_3
24 R = Cl

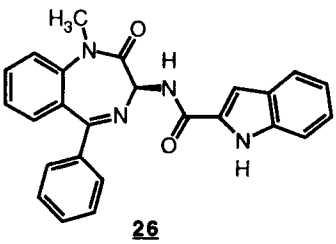
CHOLECYSTOKININ (CCK) RECEPTORS

CCK receptors are present in the brain in at least the two well-known forms, CCK-A and CCK-B. The CCK-A receptor occurs mainly in the periphery but is found in certain brain regions (31, 32). The CCK-B receptor is found throughout the brain and shows high affinity for CCK-4 (Trp-Met-Asp-Phe-NH_2), pentagastrin and the benzodiazepine antagonist L-365,260 (**25**). The affinity of the antagonist L-364,718 (MK-329, devazepide, **26**) is low. Data from both animal and human studies are accumulating to support the hypothesis that CCK plays a role in anxiety and that an antagonist of CCK will be a therapeutically useful anxiolytic (33). The activity of CCK-A selective antagonists, devazepide and L-365,031 (**27**) which possess an anxiolytic profile in the mouse black-white box paradigm, seem to implicate the type-A receptor. The B-type receptor antagonist L-365,260 appears to be only weakly active in this model. A detailed analysis of behavioral data which is relevant to subtypes A and B has been published (33).

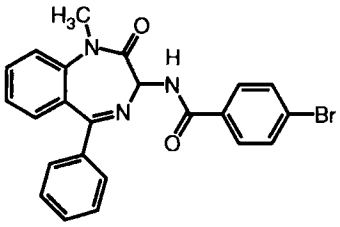
Male mice treated with devazepide (**26**) showed an insignificant preference for the black side in the black-white box paradigm (34). It was concluded that devazepide shows an anxiolytic response at these doses and that CCK antagonists may be useful as anxiolytic drugs. Related work in rats in the elevated plus-maze model implies that rats with "anxious" type of exploratory behavior possess fewer BZ-receptors in the frontal cortex compared to "non-anxious" animals. Frontal cortex CCK-8 receptor binding was upregulated by the benzodiazepine inverse agonist FG-7142 (**28**) without affecting benzodiazepine binding. Conversely, when rats were treated i.p. with CCK-8 analogs, caerulein and pentagastrin, which are anxiogenic in this model, the number of frontal cortex BZ binding sites decreased without affecting CCK-8 (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2) binding (35).



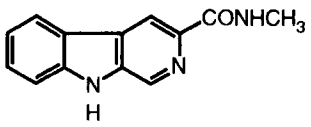
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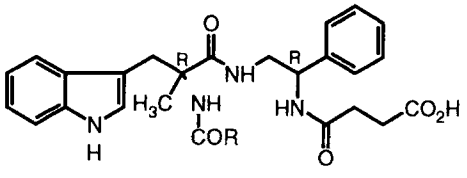


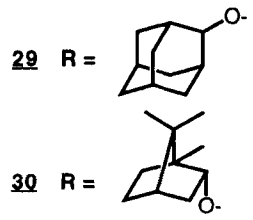
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28

The anxiolytic activity of CCK type B receptor antagonists has been further elucidated by several groups. A series of type-B receptor antagonists represented by (**29**) and (**30**) has been developed (36). The effects of long-term diazepam treatment on CCK receptor binding in rat brain has been described (37) as an extension of earlier work (38). In the same species, differences between anxious and non-anxious exploratory behavior have been correlated with brain CCK-8 and BZ receptor characteristics (35) further supporting the theory that suppression of CCK-8 excitatory activity by BZs could be at least partially responsible for their efficacy.

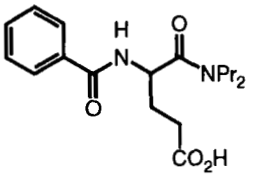




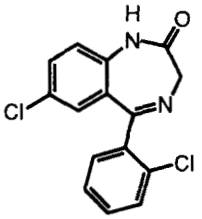
29 R =

30 R =

The importance of the CCK-8 receptor in the mouse is supported by the anxiogenic-like action of caerulein, a CCK-8 receptor agonist. This activity could be blocked by proglumide (**31**) a selective CCK-A receptor antagonist. This work suggests strongly that the type-A receptor is the relevant receptor in mice. Furthermore, panic attacks, indistinguishable from spontaneous attacks, have been induced in patients by injection of cholecystokinin tetrapeptide (25-50 μg, CCK-4) in a placebo controlled study (39). Similar results have been found with pentagastrin (0.6 μg/kg, CCK-5) in the same dose range (40). In a further extension of this work (41), panic attacks induced by i.v. adminstered CCK-4 could be blocked by the benzodiazepine, lorazepam (**32**). In the same study, CCK-8s (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2) did not induce panic attacks. However, this study is confounded by the poor ability of CCK-8s to cross the blood-brain barrier. This could imply that the type-B receptor is more important in panic attacks and perhaps also in anxiety.



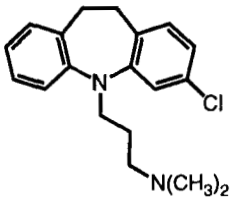
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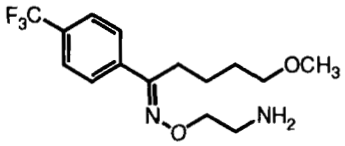
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SEROTONIN IN ANXIETY

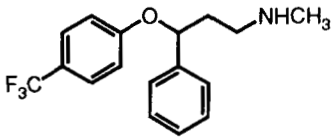
It has been suggested by clinical and laboratory investigations that changes in brain serotonin levels may contribute to anxiety. The evidence for this is currently strongest in obsessive compulsive disorders (OCD) as evidenced by the greater efficacy of selective 5-HT uptake inhibitors, clomipramine (**33**), fluvoxamine (**34**) and fluoxetine (**35**) compared to NE selective and non-selective uptake inhibitors (42).



33

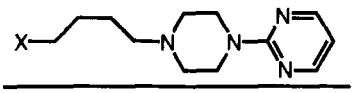


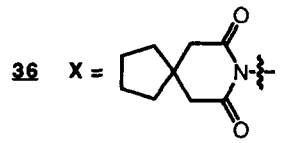
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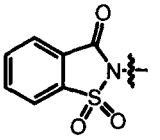


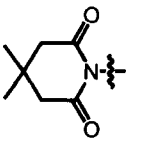
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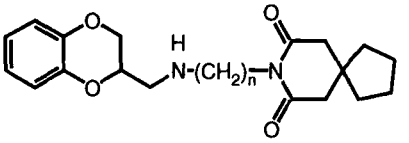
The effects of the azaspirones, buspirone (**36**), ipsapirone (**37**) and gepirone (**38**) have been extensively reviewed in 1990. In general the azaspirones interact in a complex manner at both pre- and postsynaptic 5-HT_{1A} receptors but their anxiolytic activity should not, as yet, be confidently attributed to this profile (43, 44). It has been proposed that gepirone owes its anxiolytic as well as its antidepressant activity to its ability, as a full agonist at somatodendritic 5-HT_{1A} receptors, to desensitize this autoreceptor while not affecting postsynaptic 5-HT_{1A} receptors where it is only a partial agonist (45). A detailed review of the pharmacological activities of 5-HT_{1A} partial agonist MDL 73005EF (**39**), and full agonist MDL 72832 (**40**) has been published (46). Putative receptor maps of the receptor were described. It was concluded that the partial agonist, in contrast to the full agonist, has anxiolytic potential with the possible advantage over buspirone, ipsapirone and gepirone that it is not metabolized to an α_2-adrenoreceptor antagonist which might be anxiogenic.











39 n = 2
40 n = 4

Conclusion - The improved understanding of the BZ receptor complex and the subtypes of the components may help to define the profile of an anxiolytic with fewer side-effects. Molecular biology will help to establish the relevance of the GABA and CCK receptors and their subtypes. These areas may yet yield significant therapeutic advances. Modulation of serotonin will receive continued attention which may result in incremental improvements.

Clinical data have suggested that neuropeptide Y, which has been found co-localized with GABA, may play an important role in certain CNS diseases, especially in depression and anxiety (47). This area may provide a new avenue of research.

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Chapter 2: Recent Advances In Excitatory Amino Acid Research

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Introduction - The promise of excitatory amino acids to afford both new insights into the structure and function of the central nervous system, and to provide new therapeutic opportunities continues to motivate both academic and industrial research. The continued growth in this challenging area is evidenced by the number and diversity of published research papers, reviews and symposium proceedings (1-12). In this chapter, we will highlight new and pertinent advances in excitatory amino acid (EAA) research. In particular, new excitatory amino acid agonists, antagonists and biochemical tools published over the last two years are discussed. As a reference to the discussion, a diagram outlining the complex agonist and modulatory influences on the NMDA (N-methyl-D-aspartate) receptor complex is shown in Figure 1.

Nomenclature - The recognition that quisqualic acid activates both an ion channel and a G-protein linked, IP_3 activating receptor has required the addition of the qualifying term ionotropic or metabotropic to the name quisqualate (QUIS) receptor. In part, this unwieldy nomenclature has been replaced by the redefinition of the ionotropic channel as the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropanoic acid, **1**) receptor (10). For the metabotropic receptor, the alternate term Q_p has been coined (13). However, in keeping with the tradition of naming receptors after specific agonists, the term ACPD receptor (derived from trans-1-amino-1,3-cyclopentanedicarboxylic acid (14)) is gaining in popularity (10). The influence on the classification and naming of excitatory amino acid receptors by advances made in molecular biology is not yet apparent.

Physiology - The function of EAA receptors in learning and memory has been further developed, including the ability of the NMDA receptor to mediate synaptic plasticity (15). More recently, quisqualate receptors were also found to be important in long term potentiation (LTP), an event which may be responsible for learning and memory (16). A mechanism by which the NMDA receptor might mediate neuronal maturation and synaptogenesis in the spinal cord was described (17). NMDA receptors were also shown to play a critical trophic role in the developing visual cortex of both the cat and frog (18,19). Looking beyond their function in development and memory, the relevance of EAA receptors in neurodegenerative disorders is being examined closely. The role of EAAs in Alzheimer's Disease remains an area of active study and has been reviewed recently (20). In contrast to the selective loss of NMDA receptors in Alzheimer's patients, the number of kainate receptors appear to be unaffected (21).

The number of chronic and acute neurodegenerative disease models in which EAAs are implicated continues to expand (4-6,22,23). Postmortem studies of patients with Huntington's Disease and amyotrophic lateral sclerosis show a reduction in the number of NMDA receptors observed by autoradiography. This suggests a causative role for the NMDA receptor in Huntington's disease and amyotrophic lateral sclerosis (24,25). In addition, the death and neurologic dysfunction in humans associated with the ingestion of domoic acid confirmed that kainate receptors are involved in the associated acute neurodegeneration (26). Studies both in vitro and in vivo demonstrated that EAAs participate in the neurodegeneration which develops following traumatic brain and spinal injury (27-29). Implying a more general neurotoxic role for glutamate, NMDA antagonists were neuroprotective against both metabolic and neurologic insults (30,31). In addition, mildly acidic pH (32), glycosphingolipids (33) and fibroblast growth factor (34) were also shown to be protective against NMDA specific neurotoxicity. Although the neuroprotective efficacy of NMDA antagonists has clearly been established, a paradoxical action to induce a specific, acute, and reversible neuropathology was

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reported (35). In the presence of bicarbonate ion, cysteine becomes an NMDA specific neurotoxin (36). This result is similar to earlier observations with ß-methylaminoalanine, 2,3-diaminoproprionate and 2,4-diaminobutyrate (37) . The inhibition of cysteine uptake and the neuroprotective efficacy of several antioxidants has delineated an additional mechanism underlying glutamate neurotoxicity (38). Recently, the neuroprotective action of CNQX (2) and DNQX (3) in vitro, and NBQX (44) in vivo, has firmly established that non-NMDA receptors also mediate neurotoxicity (39,40). Interestingly, the calcium channel blocker, nifedipine, was neuroprotective at micromolar concentration in vitro against chronic exposure to AMPA, kainate or quinolinate (41).

The regulatory role of EAA receptors in controlling and utilizing intracellular free calcium was reviewed (42), and the ionic dependence of acute neurotoxicity was established (43). Using fura 2-based digital imaging techniques, the spatial and temporal relationship of EAA receptor stimulation and intracellular calcium accumulation was demonstrated (44,45). Additionally, it was noted that protein kinase C may be involved in mediating calcium-dependent neurotoxicity (42,46).

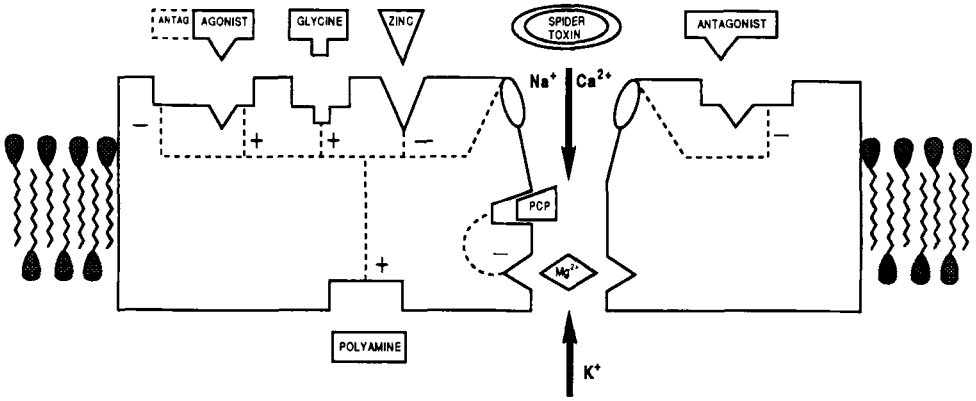


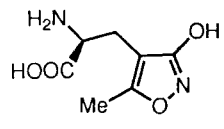
Figure 1. NMDA Receptor - Ligand Interactions. In this diagram, the NMDA receptor, with its associated ion channel and modulatory sites, is shown embedded in a lipid membrane. Agents which facilitate the operation of the channel are indicated by a plus sign and include: glutamate or NMDA (agonist), glycine and polyamines. Inhibitory agents, indicated by a minus sign, include: competitive NMDA antagonists (antagonist), noncompetitive antagonists (PCP), spider toxins and magnesium (Mg^{2+}). To accommodate unresolved questions regarding the existence of a separate antagonist binding subunit, two alternative antagonist sites are shown. In addition, the polyamine site is shown to be located on the cytosolic face of the receptor, although the actual location of the polyamine site(s) is yet to be determined.

In addition to their critical role in chronic and acute neurodegeneration and resulting neuropsychiatric disorders (4-6), EAAs have been implicated in the etiology of schizophrenia (47,48). From an understanding of the intrinsic pathways of the basal ganglia and the therapeutic action of NMDA antagonists in a range of animal models, NMDA antagonists were proposed to have utility in the treatment of Parkinson's Disease (49-52).

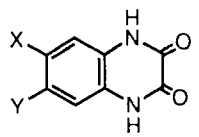
EAA receptors are also clearly implicated in epilepsy (53). Exploring both the therapeutic potential and behavioral liabilities, competitive and noncompetitive NMDA antagonists were compared in four seizure models (54). Among competitive antagonists, CGP 37849 (4) and CGP 39551 (5) demonstrated potent oral anticonvulsant activity in rat and mouse (55). Similarly, CPP (6) and CPP-ene (7) were effective in the mouse and baboon (56).

EAA receptor pathways are prominent in other areas of CNS research. Recently, AMPA antagonists were suggested to be useful in the treatment of drug addiction (57). Acting on different sleep stages, both NMDA and non-NMDA (generally AMPA and kainate) antagonists exhibit a strong

sleep promoting action in cats (58). Capitalizing on these observations, several competitive NMDA antagonists have been patented for sleep promotion and the treatment of migraine (59,60). Antidepressant activity in mice was reported for NMDA antagonists (61). When applied directly into the rat median raphe nucleus, both NMDA and non-NMDA antagonists modulate food consumption (62). Illustrating an important role for EAAs in the regulation of the hypothalamus, intrahypothalamic injection of glutamate caused a stimulatory action on rat thermogenesis (63). However, it is the influence of hypothalamic NMDA receptors on endocrine function and the onset of puberty which has received greater attention (7,64,65).

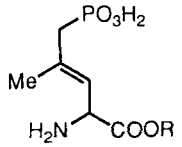


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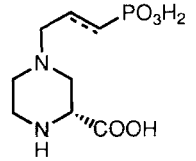
2 X = CN, Y = NO_2

3 X, Y = NO_2



4 R = H

5 R = Et



6 (saturated)

7 (E)

From the viewpoint of EAA receptor distribution (66), stimuli specificity (67,68) and neuronal plasticity (69), the role of EAAs in nociception was more clearly defined. In addition, a modulatory role of Substance P on spinal NMDA receptors has emerged (70). The ability of EAA receptors to selectively mediate spinal reflex activity was defined further (33). EAA receptors are also involved in inspiratory drive (71).

Receptor Distribution - [^{3}H]CNQX-based autoradiography has been used to localize non-NMDA receptors in both chick and rat brain (72,73). In both studies, [^{3}H]CNQX binding represented the combined distribution of kainate and QUIS receptors. Furthermore, in one study, two affinity states of the AMPA receptor were observed (72). A more extensive autoradiographic study of EAA receptors in human cortex has been described using [^{3}H]-1-(1-(2-thienyl)cyclohexyl)piperidine (TCP), glycine, L-glutamate, AMPA and kainate (74). EAA receptors have been solubilized using either ionic or non-ionic detergents. In rat pituitary and retina, quisqualate-sensitive glutamate binding has been determined (75,76). AMPA-selective binding was observed in rat brain, and a kainate-preferring site was reported in frog (77,78). The macromolecular NMDA receptor complex has been solubilized in a functional state from both rat and pig brain (79,80). A new glutamate binding protein was also extracted from pig brain (81).

Receptor Characterization - Early progress in cloning, sequencing and expressing kainate binding proteins has been followed quickly by the identification of a series of homologous non-NMDA receptor subunits (82-84). It is apparent from these results that kainate and AMPA receptors are derived from the same gene products. Those known kainate and AMPA receptor characteristics of ligand selectivity, unitary conductance and desensitization were all observed in Xenopus oocytes and cultured mammalian cells transfected with homomeric or heteromeric mixtures of receptor mRNA or cDNA, respectively. In recent cloning work, the observation of alternate gene splicing in the transcription of these glutamate-selective channel proteins is very important (85). These alternate receptor protein products, named "flip" and "flop", differ significantly in their ligand selectivity and electrophysiological characteristics. A further novel glutamate receptor subunit has been cloned recently. Although 40% homologous to previously sequenced receptor subunits, its distribution in embryonic rat CNS suggests a role in neuronal differentiation and synapse formation (86). A similar glutamate binding site has been revealed by autoradiography (87).

Although the NMDA receptor has not yet been cloned or sequenced, classical biochemical techniques have been used to define its protein composition. Using [^{3}H]azido-MK-801, and [^{3}H]azido-PCP photoaffinity labelling, receptor proteins of 120, 90, 68, 49, 40 and 33 KDa have been identified (88,89). From amino-PCP-agarose affinity chromatography, proteins of 67, 57, 46 and 33 KDa were observed (90). Antibodies raised against a glutamate binding protein recognize 71 and 63 KDa receptor proteins (91). Using these antibodies, a postsynaptic receptor location was identified. Following solubilization and affinity chromatography, a protein of 58-60 KDa selective for competitive

NMDA antagonists was isolated (92). Molecular target size analysis has suggested that the glutamate, noncompetitive NMDA antagonist, and glycine binding site-associated proteins are similar in size and are significantly different from that of the sigma recognition site (93,94). Using a kinetic analysis of antagonist action, the stoichiometry of glutamate to glycine binding has been shown to be 2:2 (95). From these data, a plausible arrangement of NMDA subunits that form the receptor complex can be derived (Figure 2). In addition to the size and multiplicity of NMDA receptor subunits, studies have also suggested a complex conformational interaction of the glutamate, competitive antagonist and glycine binding sites (96,97). The NMDA receptor is also strongly influenced by environmental factors, being downregulated by an acidic or oxidizing environment and upregulated by the converse (98,100). The activation of AMPA/kainate channels are also influenced by pH and the presence of thiophilic agents (101-103).

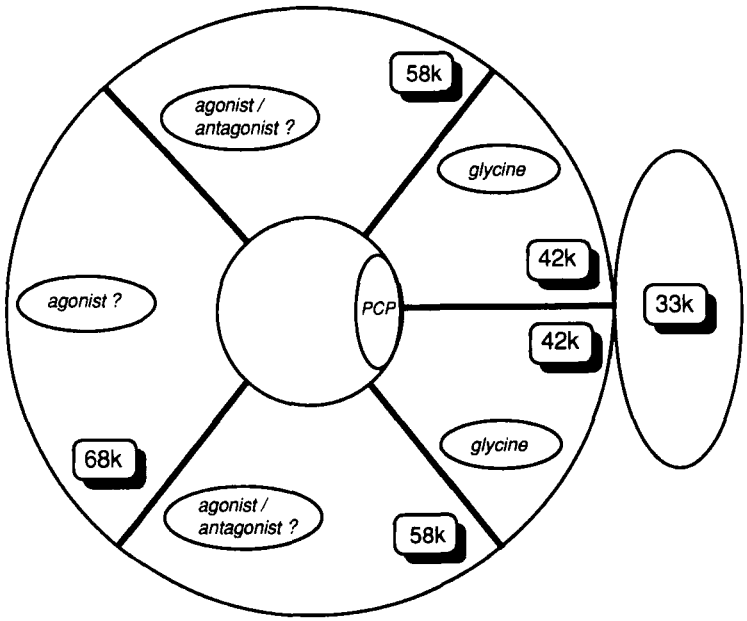
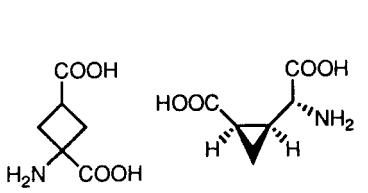


Figure 2. Hypothetical Configuration of Protein Subunits of the NMDA Receptor. From protein isolation and ligand binding site stoichiometry studies summarized under Receptor Characterization, the diagram represents a hypothetical arrangement of protein subunits and their associated ligand binding sites that may comprise the NMDA receptor complex. As such, this arrangement represents an $\alpha_2\beta_2\gamma.\delta$ subunit composition.

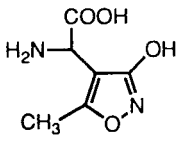
NEW PHARMACOLOGICAL AGENTS

NMDA Receptor Agonists - Conformationally restricted glutamate analogs were synthesized to help define a discrete conformation required for NMDA receptor activation. trans-1-Amino-1,3-cyclobutanedicarboxylic acid (**8**) and (1S,2R)-2-(2-carboxycyclopropyl)-D-glycine (D-CCG, **9**) are potent NMDA agonists with greater selectivity than L-CCG (104-107). The cis isomer of 3'-(methoxymethyl)-2-(2-carboxycyclopropyl)-L-glycine (c-MCG-IV, **10**) has greater NMDA selectivity than L-CCG, while the trans derivative (t-MCG-IV, **11**) is a potent and selective kainate agonist (107). From binding and electrophysiology in rat cortical tissue, α-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid (AMAA, **12**) is a highly selective NMDA agonist (108). cis-2,4-Azetidinedicarboxylic acid was suggested to be a weak or partial agonist of the NMDA receptor (109). The SAR of other NMDA and quisqualate agonists has been examined (110).



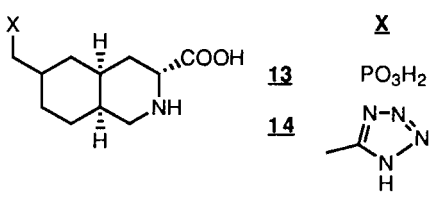
8 9

	R_1	R_2
10	CH_3OCH_2	H
11	H	CH_3OCH_2

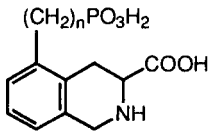


12

Competitive NMDA Antagonists - Competitive NMDA antagonists constrained within a tetrahydro-3-isoquinolinecarboxylic acid template have been reported (111-113), and are illustrated by the cis-fused decahydroisoquinoline derivatives, LY274614 (13) and LY233536 (14) (111). Other tetrahydroisoquinoline derivatives, 15 and 16, also showed significant receptor binding (112,113).

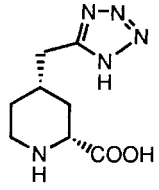


	X
13	PO_3H_2
14	

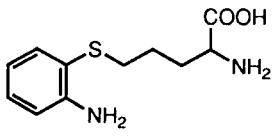


	n
15	0
16	2

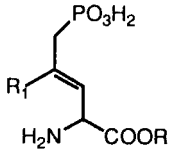
In 14 and 17, bioisosteric replacement of the phosphonic acid group with tetrazole retained potent antagonist activity (114). However, phenyl, ethyl and methylphosphinate derivatives of APV (2-amino-5-phosphonovaleric acid) and APH (2-amino-7-phosphonoheptanoic acid) had reduced affinity for the receptor (115). An ortho-aminothiophenol derivative (18) was much weaker than APH in blocking increases of cGMP in response to NMDA in rat cerebellar slices (116). A spatial model of the bioactive conformation was derived from CGS 19755 and a series of piperidine 2-carboxylic acids (117). More recently, a computer-generated NMDA antagonist pharmacophore model has been reported which provides a 3-D view of antagonist interactions, and was used to predict the receptor affinity of 14 and 15 (118).



17



18



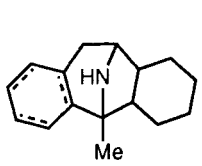
	R	R_1
19	H	C_3H_7
20	H	C_2H_4COOH

CGP 39653 (19) is the highest affinity NMDA receptor ligand yet described (119). A related compound, (E)-2-amino-4-(phosphonomethyl)hept-3-en-1,7-dicarboxylic acid (20) represents a new class of NMDA antagonist (120). The high NMDA receptor affinity of (R)-4-oxo-5-phosphononorvaline (4-oxoAPV) was suggested to result from the conformationally restricted enol tautomer (121). The significant NMDA antagonist activity of N-(E)-(4-phospono-2-butenyl)glycine showed that the phosphonoalkyl side chain could be attached through nitrogen rather than the α-carbon (122). The SAR of CPP was elaborated (123), and the enantiomers of CPP and CPP-ene were prepared (124). D-CPP-ene was effective against spontaneous and NMDA-induced depolarizations in rat neocortical slices (125), demonstrated neuroprotection in an acute subdural hematoma model in rat, and reduced

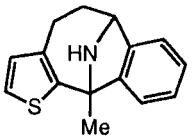
infarct volume significantly in a middle cerebral artery occlusion model in cat (126,127). CGS 19755 had NMDA-antagonist effects in rhesus monkey without ketamine-like behavioral effects (128), improved outcome after global cerebral ischemia (129,130), and reduced infarct size following cerebral artery occlusion in rat (131).

Guanine nucleotides, the most potent of which was guanosine-5'-O-(2-thiodiphosphate), were reported to act as competitive antagonists at micromolar concentrations, and may represent a regulatory mechanism for glutamate receptor activation (132).

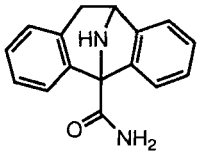
Non-competitive NMDA Antagonists - A pharmacophore model of the PCP receptor has defined the critical hydrogen bond angle and hydrophobic interactions essential for high affinity ligands (133). Evaluation of 73 dibenzo[a,d]cycloalkenimines provided a range of in vitro active compounds, although none were as potent as MK-801 in vivo (134). Other MK-801 derivatives, **21** and **22**, showed that the aromatic rings are not required for high affinity or NMDA antagonism (135). The role of C5-substitution was also examined (136). An MK-801 analog **23**, modified in both the A and B rings, retained high PCP receptor affinity (137). A hybrid structure of MK-801 and carbamazepine (ADCI, **24**) has anticonvulsant activity with reduced toxicity (138).



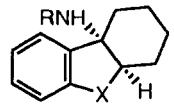
21 , 22



23



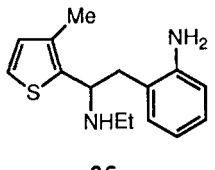
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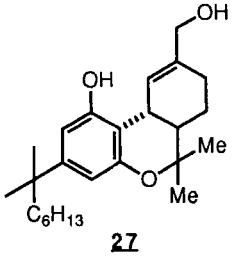
25 R = H, Me

X = CH_2, CH_2CH_2

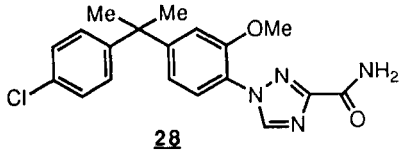
The fluorenamines and phenanthrenamines (**25**) are potent PCP receptor ligands (139-141). Flexible molecules showing significant PCP receptor affinity include the open chain thienylalkylamine (HOE 8319, **26**), 1,2-diphenylpropylamine, the major metabolite of the anticonvulsant ramacemide, and (+)-1-(1,2-diphenylethyl)piperidine (142-144). Divergent structures such as tetrahydroisoquinolines and diarylguanidines demonstrated some selectivity for the PCP receptor (145,146). Electrophysiological data show that memantine, an antiParkinson agent, had an effect comparable to MK-801 at the NMDA receptor channel (147), and was neuroprotective in vitro and in vivo (148).



26



27



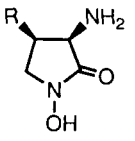
28

Other Non-competitive NMDA Antagonists - The cannabinoid (HU-211, **27**) was reported to have NMDA antagonist activity (149). A novel triazole (**28**, CGP 31358) may have activity coupled to both the transmitter recognition site and the ion channel (150). Synaptically released zinc was reported to reduce glycine binding and inhibit NMDA-mediated responses in cultured neurons, supporting the modulatory role of zinc on the NMDA receptor complex (151). The effects of ethanol on cognition and CNS development may be explained by NMDA receptor inhibition, perhaps through the glycine-site (152). Withdrawal from chronic alcohol administration reveals upregulation of NMDA receptor systems and may be responsible for seizures (153).

Peptide Antagonists - ß-Neuroprotectin, (D)-Ala-Asp-Leu-Ile-Ala-Tyr-Leu-NH2, isolated from a trypsin digest of horse cytochrome C, was found to be a potent inhibitor of [^{3}H]-TCP binding, and protected cultured hippocampal cells against NMDA-mediated cell death (154). Conantokins G and T, sleeper

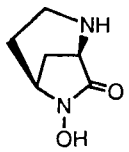
peptides from the venom of Conus geographus and Conus tulipa, were found to be partial antagonists of NMDA-induced calcium influx in rat cerebellar granule cell neurons (155).

NMDA Receptor Glycine-Site Modulators - (R)-(+)-HA 966 (**29**), a selective glycine antagonist (156), blocks NMDA-induced convulsions with no PCP-like behavioral effects (157-160), and selectively reduces the degree of NMDA-mediated, but not quisqualate-mediated brain injury (161-163). The methyl-substituted and bicyclic analogs of HA 966 (**30**,**31**) have been reported (164). These compounds further define the steric limits of glycine-site binding. 1-Amino-1-cyclobutanecarboxylic acid is also a glycine-site antagonist (165,166).

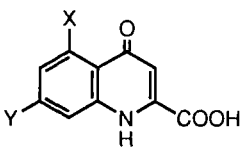


29 R = H

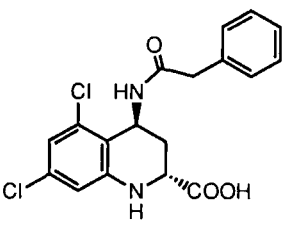
30 R = CH_3



31

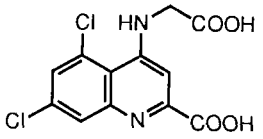


	X	Y
32	Cl	Cl
33	Br	Br
34	I	Cl

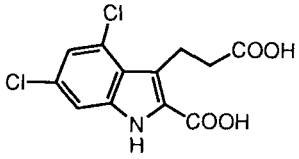


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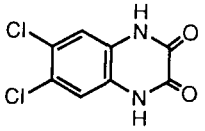
Receptor models for glycine antagonist binding were outlined (167,168). 5,7-Disubstitution of kynurenic acid (**32**-**34**) enhanced glycine-site selectivity and affinity (168). Similar glycine antagonists, such as **35** and **36**, that expand the dimensions of known steric tolerance were described (169,170). Chain length affected receptor affinity of the indole, **37**, and may be suggestive of a receptor pocket (171,172). DCQX (**38**) is a further structural lead with selective glycine antagonist activity (173).



36

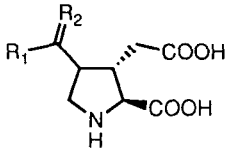


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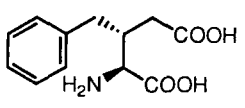
38

Polyamine Modulation of NMDA Receptor - A comprehensive review of the SAR of polyamine modulation of the NMDA receptor complex was published (174). Arcaine (1,4-diguanidinobutane) is a competitive antagonist of spermidine- and magnesium-induced [^{3}H]-TCP binding (175), or may be an inverse agonist similar in mechanism to 1,10-diaminodecane. Ifenprodil and SL 82.0715 are described as NMDA antagonists via a selective interaction with a polyamine-sensitive regulatory site on the NMDA receptor (176), although this is controversial (177). α-Difluoromethylornithine, a selective irreversible inhibitor of ornithine decarboxylase blocked NMDA-induced neurotoxicity in cultured cortical neurons, supporting the claim that NMDA-induced neurotoxicity is related to polyamine synthesis (178).

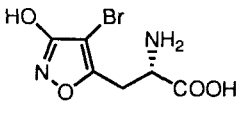


39 R_1 = Ph, R_2 = CH_2

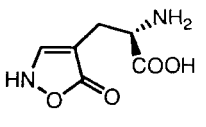
40 R_1 = Me, R_2 = NOH



41



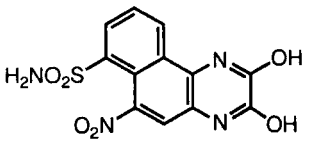
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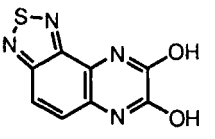
43

AMPA/Kainate Receptor Agonists - Kainic acid analogs **39** and **40** retained non-NMDA agonist activity (179), while a novel oxetane containing analog lost affinity for glutamate recognition sites (180). (2S,3R)-3-Benzylglutamic acid (**41**) is a potent kainate agonist (181). Enzymatic resolution provided the selective AMPA receptor agonist, (S)-4-bromohomoibotenic acid ((S)-Br-HIBO, **42**). Both (R)- and (S)-Br-HIBO may be inhibitors of glial uptake mechanisms which results in **42** paradoxically having less excitatory potency than the racemate (182). TAN-950 (**43**) was reported as a selective AMPA agonist (183). Systemic injection of acromelic acid A produced specific lesions of interneurons in the lower spinal cord with little or no damage to hippocampal neurons which are normally sensitive to systemic kainate (184). 2,4,5-Trihydroxyphenylalanine (TOPA), an oxidative product of DOPA, was shown to stimulate non-NMDA currents (CNQX reversible) in rat cortical neurons, and may be an endogenous excitotoxin (185).

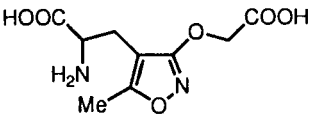
AMPA/Non-NMDA Receptor Antagonists - NBQX (**44**) is a potent, selective AMPA antagonist that protects against global ischemia, even when administered 2 hours after ischemic challenge (186). New quinoxalinediones, such as **45**, demonstrated affinity for AMPA receptors, and inhibited NMDA-stimulated [^{3}H]GABA release (187). A series of benzo-fused N-oxide quinoxalinediones was claimed as AMPA antagonists (188). α-Amino-3-(carboxymethoxy)-5-methyl-4-isoxazolepropanoic acid (AMOA, **46**) and α-amino-2,3-dihydro-2-[(3-hydroxy-5-methyl-4-isoxazolyl)methyl]-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMNH, **47**) were reported as non-NMDA receptor antagonists (108). AMOA was particularly effective against AMPA-induced current in a cortical slice preparation and was neuroprotective in striatal neurons against kainate toxicity.



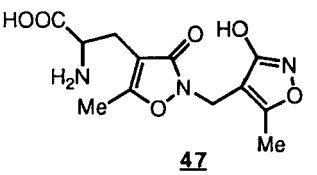
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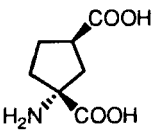
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46



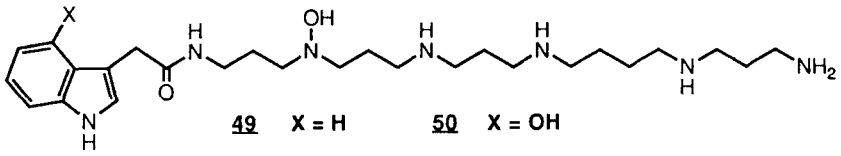
47



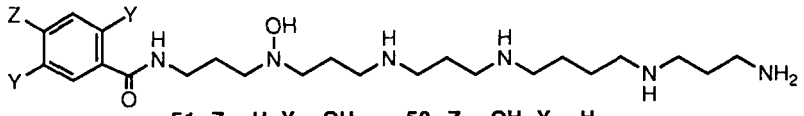
48

Quisqualate Metabotropic (ACPD) Receptor - The ACPD receptor is a discrete PI-coupled glutamate receptor which in striatal neurons and Xenopus oocytes is activated by L-glutamate, ibotenate, quisqualate, and ACPD (**48**), but not NMDA, kainate or AMPA (189). Recently, this receptor was speculated to play a key role in synaptic growth and stabilization, and may be negatively regulated by the NMDA system (10). 2-Amino-3-phosphonopropanoic acid (AP3) is a selective, potent ACPD receptor antagonist. This provided a pharmacological tool to confirm the existence of the PI-coupled ACPD receptor, and spurred an improved enantioselective synthesis of AP3 (190). Comparison of affinity of the stereoisomers revealed that the ACPD receptor prefers L-AP3 (191).

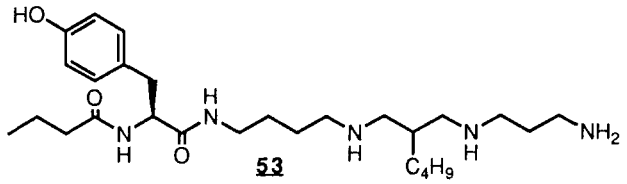
Spider/Wasp Toxins - Spider and wasp toxins that act as glutamate antagonists at the neuromuscular junction, or at subtypes of central glutamate receptors are illustrated by **49**-**53** and share a common structural theme of a polyamine side chain linked to a lipophilic head group. Argiotoxin 636 is an open channel blocker of NMDA-activated ion channels (192), and inhibited NMDA-induced seizures *in vivo* (193). Novel hydroxylamine-containing polyamines (**49**,**50**) isolated from the funnel-web spider were synthesized and were characterized as potent NMDA antagonists (194). Compound **53** is representative of about sixty synthetic analogs of philanthotoxin 433 from digger wasp venom. In this series, modifications in the polyamine moiety dramatically affected affinity at acetylcholine and NMDA receptors (195). Conceptual models of interaction with the receptor were proposed (196).



49 X = H 50 X = OH



51 Z = H, Y = OH 52 Z = OH, Y = H



53

Glutamate Release - In the striatum, the anticonvulsant lamotrigine has been reported to be neuroprotective through inhibition of kainic acid-induced glutamate release (197). A series of analogs has also been claimed to inhibit veratridine-induced glutamate release (198). Oxiracetam increases glutamate release from depolarized hippocampal slices (199). Under hypoxic conditions, glutamate release from glial cells also occurs by reversal of the electrogenic reuptake system (200).

Conclusion - The evolving diversity of excitatory amino acid receptors, the complexity of their modulatory influences, and breadth of pharmacological action have continued to both frustrate and stimulate research. However, the early successes of molecular biology in cloning and expressing receptor proteins offers the promise of more rapid and more rational drug discovery in the future. The next few years promise to be a very exciting time in excitatory amino acid research.

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Chapter 3. Progress in Antidepressant Drugs

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Introduction - Depression is the most frequently diagnosed psychiatric disorder, and despite the recent availability of several new medications for its treatment, this disease continues to be a major public health problem. Advances in antidepressant drugs were reviewed recently in this series (1). Several additional recent reviews have been published: The role of animal models of depression in the search for new drug candidates continues to be assessed (2-4). The focus of several reviews was the important role of serotonin (5HT) in depression; several mechanistically distinct classes of antidepressants all lead to enhancement of 5HT neurotransmission (5-7). This chapter will focus on the most recent progress in the discovery and development of several mechanistically defined classes of potential antidepressant drugs, and preference will be given to citations which appeared in 1990.

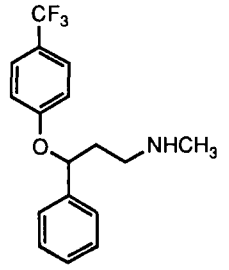
Monoamine Uptake Inhibitors - Although the first selective 5HT uptake inhibitors were described in the mid-1970s, only within the past several years have these compounds been successfully marketed as antidepressants (8). Fluoxetine (**1**) has been marketed for several years in many countries; fluvoxamine is marketed in some countries, and sertraline and paroxetine are currently being launched in multinational arenas. Many experimental approaches have demonstrated that all of these compounds are highly selective 5HT uptake inhibitors both *in vitro* and *in vivo*. Moreover, all appear to be clinically effective antidepressants (8). Although the efficacy of these compounds appears to be comparable to the tricyclic antidepressants, these selective 5HT uptake inhibitors have considerably lower affinities for neurotransmitter receptors than do the tricyclic antidepressants. Clinically, these lower affinities result in fewer anti-adrenergic, anticholinergic and antihistaminergic side effects for the new 5HT uptake inhibitors compared to tricyclic antidepressants. The principal differences among fluoxetine, sertraline, and paroxetine relate to the clinical doses and half-lives in man (9,10).

The preclinical pharmacology of fluoxetine has been reviewed (11). The absolute configurations of the enantiomers of fluoxetine have been reported previously (12), and the biochemical and pharmacological profiles of the *R* and *S* enantiomers of fluoxetine were reviewed; both enantiomers are virtually identical to the racemate as 5HT uptake inhibitors and display eudismic ratios near unity both *in vitro* and *in vivo* (13). Norfluoxetine, the *N*-desmethyl metabolite of fluoxetine, is essentially equipotent with fluoxetine as a 5HT uptake inhibitor. The homochiral enantiomers of norfluoxetine have been prepared recently and studied as 5HT uptake inhibitors (14). In rat brain cortical synaptosomes, the *R* and *S* enantiomers of norfluoxetine inhibited 5HT uptake with IC_{50} values of 484 and 54 nM, respectively. Thus, as an inhibitor of 5HT uptake, *S*-norfluoxetine is equipotent with the two enantiomers of fluoxetine, but is 9 times more potent than *R*-norfluoxetine *in vitro* (14). This increased eudismic ratio of norfluoxetine was also apparent following *in vivo* comparisons of the two enantiomers (15). Fluoxetine inhibits cytochrome P-450 which leads to some drug-drug interactions, and the most widely noted have been interactions with tricyclic antidepressants; the clinical data have been reviewed recently (16,17). Combinations of fluoxetine with monoamine oxidase (MAO) inhibitors such as tranylcypromine or phenelzine lead to a "serotonergic syndrome" in man, the symptoms of which include myoclonus, tremor, and confusion (18). This interaction probably results from both pharmacological interventions elevating the concentrations of 5HT in the synaptic cleft by mechanistically distinct avenues. This interaction between 5HT uptake inhibitors

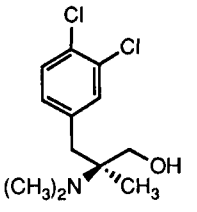
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and MAO inhibitors may be general, and has also been reported for the combination of clorgyline and clomipramine (19).

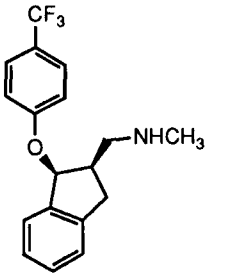
Because 5HT uptake inhibitors lead to an enhancement of 5HT neurotransmission in all 5HT neurons and brain regions, they may be useful in treating many affective and behavioral disorders. For example, clomipramine has been marketed for treatment of both depression and obsessive-compulsive disorder, and reviews have appeared on its clinical pharmacology (20,21). In a double-blind, placebo-controlled trial, sertraline was shown to be effective in treatment of obsessive-compulsive disorder and its effects resembled those seen previously with fluvoxamine, fluoxetine, and zimelidine. The selective 5HT uptake inhibitors appear to consistently perform better than tricyclic antidepressants in treatment of obsessive-compulsive disorder (22). In a placebo-controlled, double-blind study, fluvoxamine reduced the number of panic attacks and decreased avoidance behavior (23). A double-blind placebo-controlled study of citalopram in non-depressed patients with Alzheimer's disease was reported. Although this selective 5HT uptake inhibitor did not improve memory or other intellectual functions, it did improve emotional bluntness, confusion, and agitation (24). Use of fluoxetine in the treatment of obesity continues to be studied, and placebo-controlled trials suggest it produces weight loss by decreasing caloric intake (25,26). Fluoxetine and sertraline continue to be examined as adjunctive therapies in the treatment of alcoholism (27,28).



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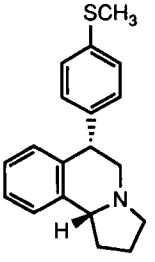


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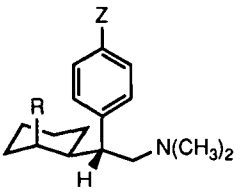


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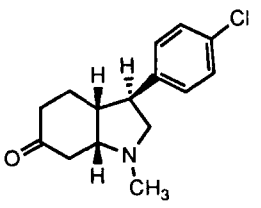
Several recent publications have described additional 5HT uptake inhibitors. JO-1017 (2) selectively inhibits 5HT uptake and has high affinities for [^{3}H]-imipramine and [^{3}H]-paroxetine binding sites in brain tissue. The compound has been examined in several rodent models which are reported to detect potential antidepressant drugs: the compound decreases escape failures in the learned helplessness test in rats and immobility times in behavioral despair tests in mice (29). Early clinical studies suggest the compound is an effective, well-tolerated antidepressant (30,31). MDL-28618A (3) is a conformationally defined analogue of fluoxetine, and the dextrorotatory enantiomer is 10 times more potent than the levorotatory isomer as a 5HT uptake inhibitor (32). The SAR and some aspects of the phar-



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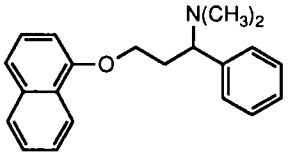


5 R = H, Z = CH_3O
6 R = OH, Z = Cl

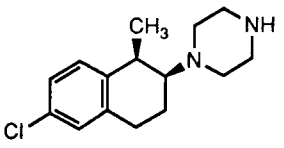


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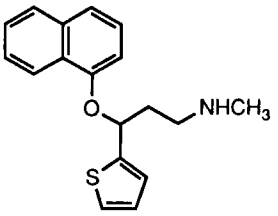
macology of McN-5652-Z (4) were reported. It is a potent inhibitor of 5HT uptake *in vitro*, and virtually all of the activity resides in the *trans*-(+)-6*S*,10b*R* enantiomer; the eudismic ratio is approximately 150. It is one of the most potent agents yet described (ED_{50} = 80 μg/kg, i.p.) in the mouse 5-hydroxytryptophan-induced head-twitch assay (33). The SAR of venlafaxine (5) was reported, and the optimal compounds possessed halogen or methoxy substituents at the 3- and/or 4-positions of the aromatic ring. Venlafaxine is a relatively nonselective inhibitor of 5HT and norepinephrine (NE) uptake *in vitro* (K_i values were 210 and 640 nM, respectively). The eudismic ratio of the enantiomers of venlafaxine is less than 3 for inhibition of either 5HT or NE uptake (34). An analogue of venlafaxine, Ro 15-8081 (6), is a nonselective inhibitor of 5HT and NE uptake, and has been studied clinically as a dual analgesic-antidepressant (35). Its bicyclic metabolite, 7, is a selective inhibitor of NE uptake, and compared to the parent compound, 6, it was considerably less potent as an analgesic agent in animal models; these data suggest that 5HT uptake inhibition plays a role in the analgesic effects of the parent compound (35). Dapoxetine (8) is a structurally distinct, selective 5HT uptake inhibitor that potently suppresses food intake in three different rodent paradigms (36). LY233708 (9) is a structural hybrid of piperazine-derived 5HT uptake inhibitors and aminotetralin 5HT1a agonists (37,38). Both dapoxetine and LY233708 are potent, selective 5HT uptake inhibitors *in vitro* and *in vivo*, and display very low or no affinities for the neurotransmitter receptors which mediate the side effects of tricyclic antidepressants. The dextrorotatory enantiomer of 10 (LY248686) equipotently inhibits both 5HT and NE reuptake *in vitro* (39). Compound 10 inhibited 5HT and NE uptake in an *ex vivo* paradigm with ED_{50} values of 12.2 and 14.6 mg/kg, p.o., indicating that the balanced *in vitro* uptake inhibition was also manifest *in vivo* (39).



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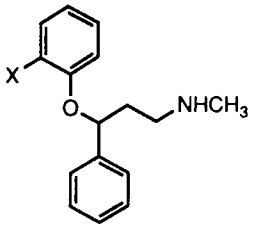


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In order to more fully understand the biochemical basis for the efficacies of antidepressant drugs, a variety of tritium-labeled ligands has been used to study the 5HT transporter, including paroxetine, citalopram, fluoxetine, imipramine, and several imipramine analogues (8). [^{3}H]-Paroxetine was shown to be superior to [^{3}H]-imipramine as a ligand for the 5HT transporter in that the former compound labels a homogeneous population of binding sites (40); [^{3}H]-paroxetine was also superior to [^{3}H]-imipramine for autoradiographic localization of the 5HT transporter (41). [^{3}H]-Sertraline was reported recently, and it selectively labels the 5HT uptake carrier (42). [^{3}H]-6-nitroquipazine is also a useful ligand for the 5HT uptake carrier. The compound labels the 5HT transporter in human platelets and rat cortical membranes with subnanomolar K_d values, and with low nonspecific binding (43-45). Because the compound has appropriate *in vivo* kinetics and penetrates the blood-brain-barrier more readily than other 5HT uptake inhibitors such as cyanoimipramine or paroxetine, 6-nitroquipazine may be a useful template for the design of 5HT uptake inhibitors which are suitable for PET or SPECT imaging (46). Two groups have described the successful synthesis of [^{11}C]-labeled citalopram for non-invasive *in vivo* studies of 5HT uptake sites in human brain using PET



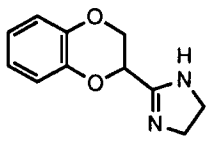
11 X = OC[^{3}H$_3$]
12 X = C[^{3}H$_3$]
13 X = [^{125}I]

imaging (47,48). [^{3}H]-Nisoxetine (**11**) and [^{3}H]-tomoxetine (**12**) are the first selective radioligands for the NE transporter (49,50). Finally, **13**, an [^{125}I]-labeled congener of tomoxetine, binds with high affinity and selectivity to the NE transporter, and enables the rapid autoradiographic visualization of NE cell bodies and fibers (51).

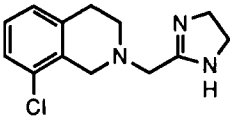
α-2 Adrenoceptor Antagonists - The medicinal chemistry and pharmacology of α-2 adrenoceptor antagonists have been actively explored in recent years. The presynaptic α-2 adrenoceptor appears to be the terminal autoreceptor of noradrenergic neurons, and blockade of these receptors represents a theoretically attractive means of increasing noradrenergic drive. Therefore, α-2 adrenoceptor antagonists and inhibitors of the NE uptake carrier (such as tricyclic antidepressants) should increase synaptic concentrations of norepinephrine by mechanistically distinct paths (52). Mianserin is an antidepressant drug whose mechanism of action may be blockade of presynaptic α-2 receptors on NE nerve terminals, resulting in increased release of NE (53). However, mianserin has multiple pharmacological actions, which complicate interpretations about the possible value of selective α-2 adrenoceptor antagonists in the treatment of depression.

The probable existence of α-2 adrenoceptor subtypes continues to receive considerable attention, and α-2a and α-2b adrenoceptors have been proposed based upon radioligand binding studies (54). Moreover, Regan and colleagues have demonstrated the existence of multiple genes which encode for α-2 adrenoceptors (55). These α-2 adrenoceptor subtypes may mediate functionally distinct processes; for example, α-2a, but not α-2b, adrenoceptor antagonists appear to block α adrenoceptor-mediated inhibition of insulin release in the rat pancreas (56,57). The design of highly selective compounds which will assist in the elucidation of these purported α-adrenoceptor subtypes continues to be a formidable objective.

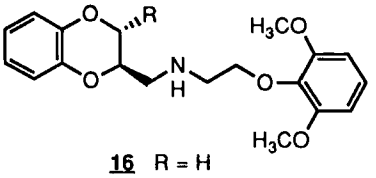
The imidazoline derivative, idazoxan (**14**), is the prototypic α-2 antagonist. It has served as a template for much of the recent α-2 antagonist medicinal chemistry. The SAR of a series of tetrahydroisoquinolinylimidazolines related to idazoxan was reported (58). In this series, some derivatives were found to be modestly selective as antagonists of [^{3}H]-idazoxan labeled sites, whereas some had selectivity as antagonists of [^{3}H]-yohimbine labeled sites in brain tissue. On the basis of radioligand binding studies, the 8-chloro derivative **15** demonstrated a 7-fold selectivity for α-2 versus α-1 adrenoceptors. Moreover,



14



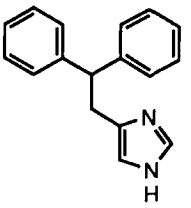
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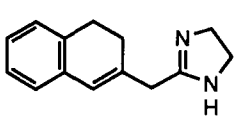
16 R = H
17 R = Ph

the compound displayed 36-fold selectivity as an antagonist of [^{3}H]-idazoxan- versus [^{3}H]-yohimbine-labeled α-2 adrenoceptors. As determined by functional studies in the guinea pig ileum, the compound was a partial agonist at α-2 adrenoceptors (58). Benzodioxane derivatives may function as antagonists at either α-2 or α-1 adrenoceptors (e.g., idazoxan or WB 4101 (**16**), respectively). Compound **17**, the analogue of **16** in which a *trans* phenyl group has been placed at position 3 of the dioxane ring, is substantially more selective than **16** for α-1 adrenoceptors. This was primarily the result of a phenyl-induced reduction in affinity for α-2 receptors, presumably due to a sterically deleterious interactions of the phenyl moiety with the α-2 receptor (59). The selectivity ratios of **16** and **17** for α-1 versus α-2 receptors were 794 and 23442, respectively. The imidazole derivative **18** is a potent antagonist of α-2 adrenoreceptors, with a pA_2 of 8.73 versus clonidine-induced inhibition of electrically simulated contractions of the guinea pig ileum. Importantly the α-2/α-1 adrenoceptor selectivity ratio was 81. Finally, the compound was also reported to be a weak inhibitor of the presynaptic NE transporter; by promoting the release of NE *and* blocking its reuptake, such a compound would be expected to enhance noradrenergic

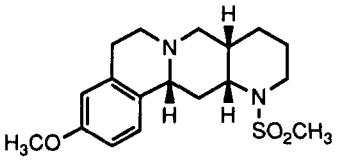
function markedly (60). Napamezole (**19**) has been previously described as an α-2 adrenoceptor antagonist with modest selectivity for α-2 versus α-1 adrenoceptors. Full reports on the *in vitro* (61) and *in vivo* (62) pharmacology of napamezole were recently published; *in vitro* studies demonstrated that napamezole weakly inhibits 5HT reuptake. However, *in vivo*, the compound appeared to block α-2 adrenoceptors and inhibit 5HT uptake at comparable doses, suggesting that the compound could affect two key neurotransmitters simultaneously. The tetracyclic compound **20** is a structural hybrid between rauwolscine and the previously reported α-2 adrenoceptor antagonist WY 26703 (**21**). The 8a*R*,12a*S*,13a*S* isomer is over three orders of magnitude more potent than its optical antipode, and the α-2/α-1 selectivity ratio of the compound is in excess of 15000 (63,64).



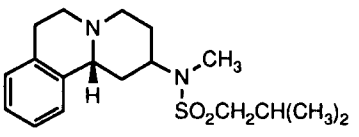
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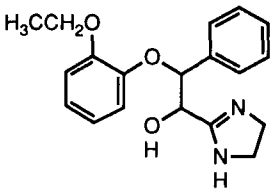
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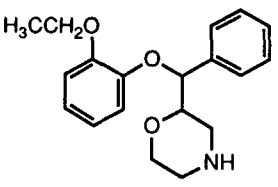
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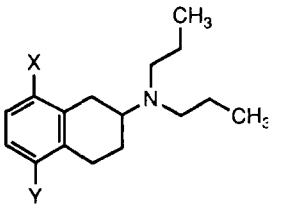
Compound **22**, a structural hybrid of reboxetine (**23**) and idazoxan, was synthesized to combine the NE uptake-inhibiting properties of the former drug and the α-2 adrenoreceptor blocking properties of the latter drug. Unfortunately, **22** and several analogs were not active either as α-2 receptor antagonists or as norepinephrine uptake inhibitors (65).

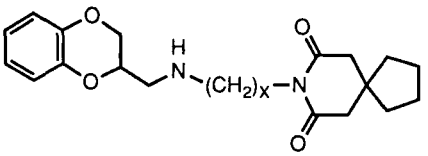
5HT1a Agonists - A general discussion of the medicinal chemistry of 5HT1a agonists can be found in Chapter 11 of this volume (66). Recent clinical studies have found that buspirone, gepirone, and ipsapirone are effective antidepressants (67-72). These compounds appear to exert their anxiolytic effects by their actions as 5HT1a partial agonists; moreover, it has been suggested that the same molecular action accounts for their more recently discovered antidepressant activities. However, an alternative mechanism for their antidepressant actions has been proposed. These compounds are metabolized extensively (73,74) to 1-(2-pyrimidinyl)piperazine (1-PP). This metabolite has little affinity for 5HT receptors (73), and it is not thought to be involved in the anxiolytic effects produced by administration of the parent drug (75). However, based upon its pharmacological actions, the metabolite may be involved in the antidepressant actions of these structurally related 5HT1a agonists.

1-PP is a relatively potent α-2 adrenoceptor antagonist (73,76-78). In blood and brain of animals treated with buspirone or gepirone, 1-PP levels were higher and persisted longer than parent drug (73). In blood of humans treated with buspirone, the metabolite 1-PP is present at markedly higher concentrations than buspirone (73). *In vivo* blockade of α-2 adrenoceptors occurs after buspirone or gepirone administration, due to the formation and persistence of the metabolite 1-PP (76,79-82). As described in a previous section of this

chapter, a variety of α-2 adrenoceptor antagonists are under development for the treatment of depression (83). Thus the pharmacological actions of the metabolite 1-PP complicates mechanistic interpretations relative to the antidepressant effects of presently available 5HT1a partial agonists. Further studies with additional 5HT1a receptor agonists, particularly with agonists of other structural types that do not liberate pharmacologically active substances, should clarify the mechanisms involved in the antidepressant actions of these drugs.

Some animal studies support the hypothesis that 5HT1a receptor agonists have intrinsic antidepressant effects. For example, 8-hydroxy-2-(di-*N*-propylamino)tetralin (8-OH-DPAT, **24**), a prototypic full agonist at 5HT1a receptors, was active in a learned helplessness behavior paradigm in rats (84). This model is often considered to be predictive of clinical antidepressant activity. 8-OH-DPAT, as well as piperazine-derived 5HT1a partial agonists, reduced immobility time in the forced swim test in rats (85). These behavioral studies with 5HT1a receptor agonists in rats support earlier findings (86,87). The 8-methylthio analog (**25**) of 8-OH-DPAT was reported to be more potent than 8-OH-DPAT itself (88). The 5-fluoro derivative (**26**) of 8-OH-DPAT was reported to be a relatively impotent *antagonist* of 5HT1a receptors (89).

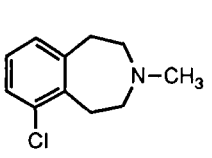


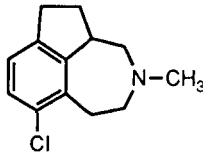


24 X = OH, Y = H
25 X = SCH_3, Y = H
26 X = OH, Y = F

27 X = 4
28 X = 2

The benzodioxane derivative MDL 72832 (**27**) has been previously reported to be a partial agonist at 5HT1a receptors (90). In a variety of assays, the eudismic ratio was approximately 32, and the levorotatory isomer was the more potent enantiomer (90,91). A homologue of this compound, MDL 73005EF (**28**), is also a potent 5HT1a partial agonist and is active in animal models of anxiety (92). Structural modification of the α-2 adrenoceptor antagonist **29** (SKF 86466) resulted in **30**, which had relatively high and balanced affinities for both α-2 and 5HT1a receptors (pK_i values were 8.1 and 7.6, respectively (93). In addition to 5HT1a agonists, preliminary clinical trials suggest that the 5HT2 receptor antagonists ritanserin (94) and nefazodone (95) may have clinical efficacy in the treatment of depression.

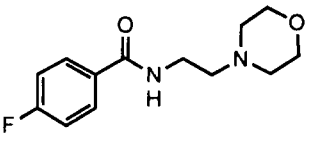




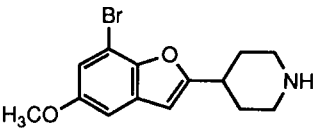
29 **30**

Other mechanistic approaches - Inhibitors of MAO have been used for more than 30 years in the treatment of depression. Because of potentially lethal hypertensive crises following ingestion of foods containing sympathomimetic amines, MAO inhibitors are used relatively infrequently in the treatment of major depression (96). However, MAO inhibitors currently used to treat depression in the U.S. are irreversible inhibitors, and are relatively nonselective for the two isozymes of MAO, type A and type B. Moclobemide (**31**), a reversible inhibitor highly selective for type A MAO, was recently introduced to the marketplace in some countries (97). Other selective and reversible MAO-A inhibitors, including brofaromine

(32), are under development (98). The fact that these compounds appear to be effective antidepressants, without the risk of severe cardiovascular side effects that plague the older MAO inhibitors, may lead to a more widespread utilization of MAO inhibitors (99).

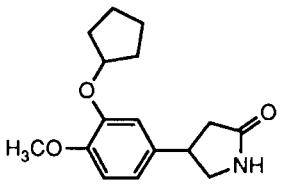


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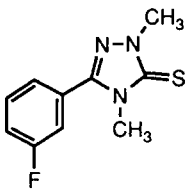


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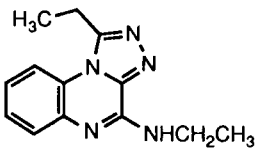
Rolipram (33) is a prototypic inhibitor of the type IV, cGMP-insensitive phosphodiesterase (PDE), an isoform of phosphodiesterase that appears to be particularly important in modulating cAMP metabolism in the CNS (100-102). Rolipram has demonstrated antidepressant efficacy in over 14 double-blind clinical trials (103-105). An SAR study of rolipram, and a topographical model of its binding site on the phosphodiesterase isozyme, were presented (106). For a wide range of rolipram analogues, there were highly significant correlations between the potencies of compounds to antagonize reserpine-induced hypothermia in mice or induce head twitches in rats, and their potencies as antagonists of [^{3}H]-rolipram binding *ex vivo* (107). These data, when coupled with the strong evidence that [^{3}H]-rolipram binds specifically to the PDE isozyme, lend further support to the hypothesis that the behavioral effects of rolipram are mediated by inhibition of type IV, cGMP-insensitive PDE (108).



33



34



35

MDL 26,479 (34) does not inhibit monoamine uptake carriers *in vitro*, but it does antagonize Ro 4-1284 induced hypothermia and reserpine-induced ptosis in mice, effects usually seen with potent monoamine uptake inhibitors (109). When given chronically, both 34 and desipramine attenuated NE enhancement of inhibitory responses to GABA on Purkinje neurons. Unlike desipramine, acute treatment with 34 decreased noradrenergic enhancement of GABA inhibition, suggesting 34 might have a faster onset of antidepressant action clinically (109).

Based upon animal studies, substances that reduce neurotransmission at the NMDA (*N*-methyl-*D*-aspartate) receptor complex (110) may represent a new class of antidepressant drugs. A variety of NMDA modulators mimicked the effects of antidepressant drugs in reducing the duration of immobility in a forced-swim test in mice, including 2-amino-7-phosphonoheptanoic acid (AP-7), a competitive NMDA receptor antagonist; dizolcipine (MK-801), a noncompetitive NMDA receptor antagonist; and 1-aminocyclopropane-carboxylic acid, a partial agonist at strychnine-insensitive glycine receptors (111). A series of 4-amino-[1,2,4]-triazolo-[4,3-a]-quinoxalines reduced immobility in Porsolt's behavioral despair model in rats, a preclinical model designed to detect antidepressants (112). These compounds were potent adenosine A1 and A2 receptor antagonists, and this was presumed to be the biochemical mechanism of their behavioral effects; 35 was one of the optimal compounds (112). Captopril exhibited activity comparable to imipramine in the learned helplessness paradigm in rats (113). Moreover, a variety of anecdotal clinical reports

suggest that captopril exhibits mood-elevating and antidepressant properties in man (114). Determination of whether ACE inhibitors, or the newly developed angiotensin II receptor antagonists (115), can be used to treat affective disorders will require carefully controlled, prospective clinical trials.

Conclusions - The decade of the 1990s will witness the introduction of a wide variety of additional antidepressant drugs. Many of these compounds will be advances in that they will produce fewer side effects than tricyclic antidepressants. However, development of compounds which produce therapeutic effects more rapidly, or in greater percentage of patients, than tricyclic antidepressants remain elusive goals. Discovery of such compounds will probably require a more sophisticated understanding of the fundamental biochemical aberrations which underlie depression.

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Chapter 4. Diversity of Neuronal Calcium Channels

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Introduction - One of the distinguishing characteristics of neurons is their excitability due to the presence of voltage-dependent ion channel proteins in neuronal membranes. Recent work has indicated that ion channels that are selectively permeable to Ca^{2+} are abundant in neuronal tissue. The abundance of voltage-dependent Ca^{2+} channels coupled with a growing appreciation of the diversity of neuronal Ca^{2+} channels has piqued interest in specific neuronal Ca^{2+} channels as targets for pharmacological intervention. In this review, we discuss recent work that explores the characteristics of neuronal Ca^{2+} channels rather than those of heart and smooth muscle which have been the main targets of previous pharmaceutical research efforts.

CLASSIFICATION OF NEURONAL Ca^{2+} CHANNELS

The current classification scheme of neuronal voltage-dependent Ca^{2+} channels grew out of earlier work in heart and smooth muscle and reflects both the similarities and differences in the voltage-dependent Ca^{2+} channels found in these tissues. While our intent is to describe the current state of knowledge of Ca^{2+} channels in central neurons, much of the recent work directed toward classification of neuronal Ca^{2+} channels is based on electrophysiological recordings using patch-clamp techniques from peripheral neurons of either whole cell ionic currents or single channels (1). Calcium channels in both central and peripheral neurons have been divided using these techniques into two categories based on the level of membrane depolarization required for activation: low voltage-activated (LVA) and high voltage-activated (HVA) channels.

A simplified scheme for the features of neuronal Ca^{2+} channels is shown in Table 1. There are important exceptions to several of these features and controversy to others. Some of these points will be elaborated below.

Table 1. Classification of Voltage-Dependent Ca^{2+} Channels

	Low voltage-activated	High voltage-activated	
Channel Class	T	L	N
Depolarization required for activation	Weak	Strong	Strong
Inactivation Kinetics	Transient	Sustained	Transient*
DHP*-sensitive	NO	YES	NO
ω-CgTx GVIA*-sensitive	NO	YES*	YES
Tissue	Neuronal & non-Neuronal	Neuronal & non-Neuronal	Neuronal

*see text

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Low voltage-activated channels - There is general agreement regarding the features that define LVA, or T-type, voltage-dependent Ca^{2+} channels. These channels are found in neurons from a variety of brain areas including cortical (2-4), midbrain (5-7) and hindbrain regions (8,9), and are also found in non-neuronal tissues including several types of muscle and a variety of additional peripheral cells. The distinguishing physiological features of T-channels are their low-voltage threshold of activation and transient duration of opening due to rapid inactivation. Thus, T-channels are opened during relatively small depolarizations from normal resting potential and turn off with a time course in the tens of milliseconds following activation. T-channels in both neurons and non-neuronal cells are generally resistant to dihydropyridines (DHPs) and the other familiar classes of Ca^{2+} channel therapeutics although this statement is somewhat clouded by recent work on several neuronal types that indicates sensitivity of T-channels to flunarizine and nicardipine (7,10). Other compounds have also been reported to block T-channels in neurons (eg., phenytoin (2), amiloride (11), and octanol (12)). However, none of these substances has proven to be sufficiently specific for T-channels to serve as a useful tool across cell types.

High-voltage activated channels - As indicated in Table 1, HVA channels in neurons have been further divided into two categories. The first category, L-channels, consists of the channels that have properties similar to the DHP-sensitive voltage-dependent Ca^{2+} channels found in heart and vascular smooth muscle. A larger degree of depolarization is necessary to activate L-channels than T-channels. Once opened, L-channels show a sustained response which can last for many hundreds of milliseconds. L-channels in central neurons are sensitive to DHP agonists and antagonists (4, 13-19). These features of L-channels are similar across different neuronal cell types (2-4, 13, 20-22). However, there is variability in the kinetics of L-channel responses that makes strict identification problematic especially in regard to separation from the other class of HVA channel (17,18)

The second broad class of HVA Ca^{2+} channels, named N-channels, was first described in peripheral neurons (13-15). N-channels, like L-channels require stronger depolarizations for activation than are required for LVA channels. N-channels have been distinguished from L-channels on the basis of a more transient response and lack of sensitivity to DHP antagonists and agonists. The time constant for inactivation of N channels is between that for the transient LVA and the sustained responses characteristic of L-channels. However, strict separation of N- and L-channels is difficult because of the overlap of depolarization levels needed for activation; thus, it is difficult to adjust stimulation parameters to activate only one of these channel types when recording from whole cells. The picture is further complicated by the differences in kinetics in N-channels between different neuronal types (4,14-16,18,22-24). Thus, both N- and L-channels kinetics differ between different neuronal cell types.

The present state of pharmacological study of N-channels does not fully clarify the picture regarding identification of HVA channels. The marine cone snail venom, ω-Conotoxin GVIA (ω-CgTx GVIA), has been used as a tool to separate HVA from LVA channels (25). There is not general agreement regarding specificity of ω-CgTx GVIA for L- or N-channels. In some cells, the actions of ω-CgTx GVIA have not been found to be helpful in separating L- from N-channel responses (25,26). However, other investigators report that ω-CgTx GVIA appears to block N-channels, since the sustained, DHP-sensitive HVA component in these cells was not sensitive to ω-CgTx GVIA block (17,18). It is clear that ω-CgTx GVIA blocks only a portion of HVA currents recorded in most neuronal types (27). In some neurons, DHP agonist action is more potent on the portion of HVA current that is not affected by ω-CgTx GVIA, indicating a separation between DHP-sensitive L -channels and N-channels by the toxin (17-19). Thus, while ω-CgTx GVIA-sensitivity may be useful in some neurons as a pharmacological label for N-channels, strict classification using this toxin remains confusing and will be clarified only when pharmacological data are available for channels for which amino acid sequences have been solved (infra vide).

FUNCTIONS OF NEURONAL Ca^{2+}CHANNELS

The present state of understanding of the functions of the subtypes of neuronal Ca^{2+} channels has been severely limited because of the paucity of specific non-L-channel pharmacological tools. However, the physiological properties of channel subtypes and work with the available pharmacological agents suggest differential functions for the channel subtypes.

T-channels have been proposed to be involved in the control of spike firing patterns in a number of different neuronal cell types (5-9, 28, 29). These channels are proposed to be responsible for the low-threshold spike that is active near normal neuronal resting potentials. The

voltage range in which T-channels are opened and closed is near resting potential and in the range for interactions with other conductances that play a role in controlling neuronal firing (30). However, a more complete understanding of the exact role of T-channels in firing patterns and the generality of that role awaits the development of specific pharmacological tools (31). One therapeutic link to T-channels has been suggested by the actions of anticonvulsants that are useful in petit mal epilepsy and block T-channels in thalamic neurons (32).

The role of L-channels in normal neuronal function is not clear despite the availability of pharmacological tools for these channels (DHP agonists and antagonists). In the studies mentioned above, DHPs have potent effects on neuronal L-channels; however many of these electrophysiological studies employ ionic conditions in which Ca^{2+} channel activity is isolated. In many assays of neuronal function, DHPs do not have a robust effect. Thus, while the role of L-channels has remained little understood, the sustained Ca^{2+} influx has been proposed as involved in triggering Ca^{2+}-dependent biochemical processes under depolarizing conditions in which L-channels would be expected to be activated (33).

N-channels have been proposed to be involved in neurotransmitter release based on the effects of ω-CgTx GVIA on neurotransmitter release or synaptic transmission in both peripheral and central synapses (16,34,35). Since DHP antagonists are not effective in these systems (but are in others, ref. 36), the actions of ω-CgTx GVIA have been attributed to the block of N-channels. These results imply that N-channels are located near release sites on nerve terminals (although also present on neuronal cell bodies since both macroscopic currents and single N-channels can be recorded there). Recent studies have shown that Ca^{2+} currents can be recorded directly from nerve terminals in two preparations in which the size of the terminal is amenable to recording. In these systems, two components of HVA-type Ca^{2+} currents have been recorded which can be distinguished from each other by susceptibility to ω-CgTx or DHP block (25,37,38). In the chick cilary ganglion, synaptic transmission is blocked by ω-CgTx GVIA, but not nifedipine, suggesting a role for the ω-CgTx -sensitive Ca^{2+} channel in synaptic transmission in this system (38).

DIVERSITY OF CALCIUM CHANNELS

There are general similarities of channels within the three classes discussed above. However, the overiding theme from many of these studies is that such a simple classification scheme does not do justice to the differences found within the classes. This is particularly true for N-channels which, to a certain extent, have been classified as any HVA channel that is insensitive to DHPs. Although, ω-CgTx GVIA has been useful in some studies as a label of N-channels, as mentioned above, the effect of ω-CgTx GVIA on L-channels is disputed and there may be subtypes of N-channels that are ω-CgTx GVIA-insensitive (18). It is clear that N-channels (as well as T- and L-channels) have different kinetic properties in different neuronal cell types. It remains to be seen whether these differences reflect molecular differences between gene products of a large family of related proteins within the T,L,N classification system. There has been progress in the resolution of this issue during the past several years on two main fronts: studies on the structure and molecular biology of Ca^{2+} channels in neurons and the work in the development of new Ca^{2+} channel pharmacological tools from animal venoms.

MOLECULAR PROPERTIES OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

Structure of L-Type Voltage-dependent Ca^{2+} Channels - Biochemical characterization and primary structure determination of voltage-sensitive Ca^{2+} channels has proceeded rapidly in recent years (39-45). Skeletal muscle (skm) T-tubule membranes are the richest source of 1,4-DHP receptors (46,47) and have consequently served as a model for structural studies of the L-type Ca^{2+} channel (39,40,48). Purified high affinity 1,4-DHP receptors from this tissue are a complex of five distinct subunits, α_1, α_2, β, γ, and δ (fig. 1). The α_1 subunit (M_r ~175 kDa) is unglycosidated and contains the multiple phosphorylation sites. The α_2 subunit is a heavily N-glycosidated polypeptide (M_r ~145 kDa) which is appended by disulfide bonds to another glycopeptide, the δ subunit (M_r 24-27 kDa). The remaining β (M_r ~54kDa) and γ (M_r ~30kDa) subunits are also glycoproteins where the β protein, like the α_1 subunit, is a substrate for protein kinase. Strong detergents are required to separate subunits from the tightly associated intact complex (49,51). Covalent labeling studies with hydrophobic (49) and Ca^{2+} antagonist (49-52) photoaffinity reagents located drug binding sites and described the intramembrane ordering of subunits. The

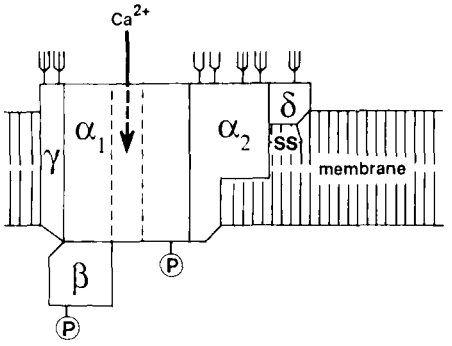


Figure 1. Subunit structure of the rabbit skeletal muscle Ca^{2+} channel with deduced disulfide bonds, glycosidation sites and phosphorylation sites.Reproduced with permission from W.A. Catterall et al. (ref. 42). © 1988 by the AAAS

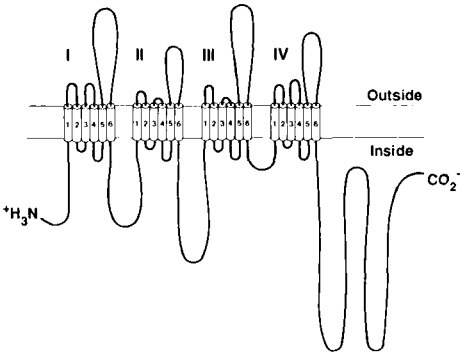


Figure 2. Proposed transmembrane arrangement of the α_1 subunit of the rabbit skeletal muscle Ca^{2+} channel. Reproduced with permission from W.A. Catterall et al. (ref. 42).© 1988 by the AAAS

DHP and phenylalkylamine recognition sites, labeled by [^{3}H]-azidopine and [^{3}H]-LU49888 (fig.3) respectively, are present on the α_1 subunit. Phosphorylation and glycosidation patterns along with selective hydrophobic photoaffinity labeling with [^{125}I]-TID (fig.3) clarified the transmembrane disposition of the intact channel complex.

The primary amino acid sequence of the α_1, α_2, β and γ subunits from skeletal muscle (53-56) and the α_1 subunit from cardiac muscle (57) have been deduced from full length cDNA. Hydropathicity profiles (58) generated for each subunits (53-56) provides predictions in accord with the subunit model generated by biochemical techniques. Of critical importance is the structure of the α_1 subunit in skm. The open reading frame of the α_1 subunit cDNA encodes a 1873 amino acid protein (212 kDa) which is related to the α subunit of the voltage-dependent Na^+ channel (1820 aa) from rat brain with a sequence homology at 29% identical and 55% with conservative replacements (53,59). Similarity matrix analysis, comparing the α_1 subunit to itself, defines four repeated domains with strong sequence homology. A model for the overall fold and transmembrane topology (fig. 2) has been developed (53) and is similar to that proposed for the voltage-dependent Na^+ channel (60). The homologous repeats (I - I V) contain six hydrophobic (α–helical) membrane-spanning elements, S1-S6. The 24 α-helical elements are connected by variable length cytoplasmic (e.g. I S 6 -> I I S 1) and extracellular (e.g. I I I S 5 -> I I I S 6) linkers. The S2 regions are negatively charged and the S4 regions contain positively charged amino acids (Lys/Arg) every third (or fourth) residue. The repeated domains (I - I V) are proposed to form four helix clusters organized around a central cation-conducting pore which is lined with four S2 segments (40,44,61). The S4 helix is a highly conserved structural element found in voltage-sensitive Na^+, K^+ and Ca^{2+} channels (42,52,59,62) and is believed to participate in voltage-dependent activation of these channels (42,43,63,64). At resting potentials the S4 helix is constrained to an ion-pairing arrangement with negatively charged residues on other transmembrane sections. Depolarization induces each S4 helix to rotate (~60°) and propagate outward (~5Å) along the helix axis. This movement conserves the register of the ion-pair interactions to S4, transfers several units of gating charge across the lipid bilayer and produces a conformational distortion leading to channel activation (65). Unlike the α_1 subunit, the functional importance of the α_2, β and γ subunits is currently unclear. However regulatory roles for these proteins have been proposed (39,57,66-69).

Several strategies have been used to reconstitute voltage-sensitive Ca^{2+} channel activity in exogenous systems. These include stable genetic transfection of cultured cell lines (68), microinjection of α_1 subunit cDNA encoded expression plasmids into myotubes of dysgenic skeletal muscle (70-71), mRNA injected *Xenopus* oocytes (57,72-75) and reconstitution of purified channels into phospholipid vesicles and lipid bilayers (76-78). These systems facilitate

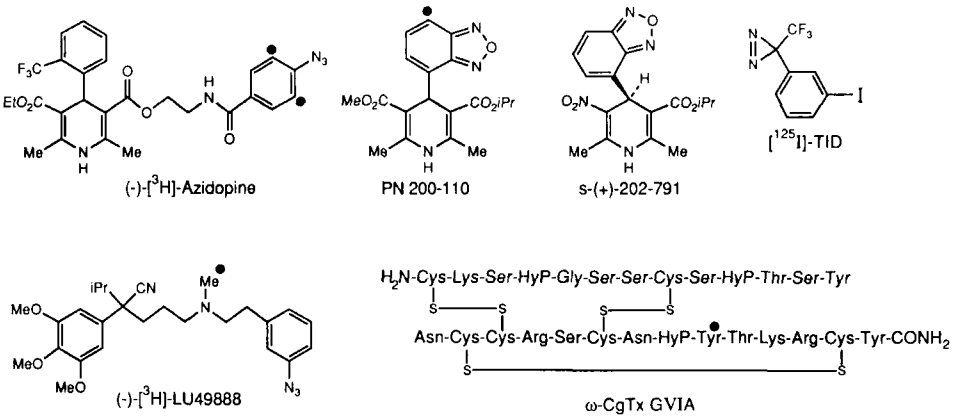


Figure 3. Important ligands and photoaffinity labelling reagents for voltage-sensitive Ca^{2+} channels. The mark (•) indicates the position of the radio label. Amino acid three letter code used. HyP is hydroxyproline.

functional characterization and genetic manipulation of target channels and subunits (40,42,79). In this way the function of the α_1 and other subunits have been investigated (39,57,68-71).

STRUCTURAL CHARACTERIZATION OF NEURONAL VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

There is compelling evidence for at least three discrete Ca^{2+} channel types in neurons (33,80-83). Though a structural view of these channels has developed more slowly than that for the skm DHP-receptor, information on the molecular diversity and biochemistry of neuronal L and N-type channels is becoming available. These studies, in time, will accurately elaborate on the current N,L and T classification

L-Type Ca^{2+} Channel: Structure - Pharmacological (84) and electrophysiological (82,85) data demonstrate that DHP-sensitive L-type channels found in neurons are distinct from those present in skeletal muscle. In accord with these studies are the observations that skm α_1-subunit cDNA does not hybridize to brain mRNA under moderate stringency conditions (54) and that neuronal and skm L-channels are likely to be encoded by separate genes since mice with muscular dysgenesis possess L-channels in sensory neurons but not in skm (86). Yet sufficient pharmacological and molecular similarity does exist such that sequence and biochemical data along with affinity reagents generated during the skm studies provided a departure point for neuronal studies.

High affinity binding sites for the dihydropyridines and phenylalkylamines in brain are well characterized (39,40,87,88) and the photoaffinity labels (-)-[^{3}H]- azidopine, and (-)-[N-methyl-^{3}H]LU49888 (fig 3.); have been used to mark each of these receptors present in guinea-pig hippocampus (89). Both photoreactive probes show typical receptor binding profiles and upon irradiation label a 195kDa protein. This protein is a likely candidate for the α_1 subunit of the DHP-sensitive Ca^{2+} channel in brain. The phenylalkylamine reagent identified a second peptide at 265 kDa. This peptide could be an element of the T-type Ca^{2+} channel since low-threshold T-currents in hippocampal neurons are blocked by phenylalkylamines (2,10).

It is evident that the α_2 subunit is conserved across tissues (54,90-95). Monoclonal antibodies raised against rabbit skm microsomal membranes (MANC-1, MANC-3) and shown to recognize $\alpha_2\delta$ subunit of the skm channel, immunoprecipitated 64% and 80% of the [^{3}H]PN-200-110 prelabelled DHP-sensitive Ca^{2+} channels from whole rabbit brain (94) and hippocampus respectively (95). The immunoprecipatant contained proteins with electrophoretic properties ($M_r(\alpha_1)$ 175 kDa, $M_r(\alpha_2\delta)$ 166 kDa, $M_r(\alpha_2)$ 142 kDa, $M_r(\beta)$ 57 kDa) identical to the α_1,$\alpha_2\delta$ and β subunits present in skm. Northern analysis of brain mRNA with cDNA probes designed from skm sequence data reveal an α_1 (57,96-98), α_2 (54) and β (55,99) but not a γ-like subunit (56) transcript. These data taken together support the proposal of an oligomeric structure for the L-channel in brain containing at least α_1. $\alpha_2\delta$ and β subunits.

	rbA-65	rbB-8	rbC-61	rbD-55	cardiac DHP
rbB-8	91	---	---	---	---
rbC-61	50	55	---	---	---
rbD-55	55	51	76	---	---
Cardiac-DHP	51	64	97	72	---
skm-DHP	47	60	76	75	66

Figure 4. Homology between Cloned Brain Ca2+ Channel sequences - cDNA clones are identified by rb class and by clone number as originally described in ref. 96. Analysis based on partial cDNA sequence data Reproduced with permission from T.P. Snutch et al., (ref. 103,104).

The diversity of DHP-sensitive Ca^{2+} channels is apparent from the isolation of tissue-specific isoforms of the α_1 subunit. Transcripts of varying size and unique sequence have been reported from a variety of tissues including brain (57,96-98,100,101). Selected and cloned α_1-cDNAs from rat brain (rb) can be organized into four classes (class rbA, rbB, rbC & rbD) based on characteristic hybridization to brain mRNA . Genomic Southern analysis showed rbA through D were encoded by four distinct genes (96). While rat brain cDNA classes A and B are more closely related to each other than to rbC and rbD, classes C and D are homologous to one another and to the cardiac and skm DHP receptors (see fig.4,103,104). In general, rbA-D exhibit strong homology within repeated domains I - IV (~90%) but show major differences in the C-terminal cytoplasmic domain and in the cytoplasmic loop region between repeats I I and I I I. In addition, subtle variations of charged residues in S4 regions exist. Such modifications could provide unique voltage-gating properties to each of these molecules. Using heart, muscle and endocrine derived tissues, PCR was employed to amplify cDNA sequences recognized by primers designed from skm and cardiac α_1 subunits (98). A comprehensive scheme (fig. 5) categorizes all known α_1 subunit cDNA clones into five non-allelic groups (CaCh 1-5). Further diversity within groups 1,2 and 3 arises from 2-4 different splicing arrangements (a,b,c,d) of pre-mRNA. Two interchangable exons coupled with a choice of two alternate splice sites produces 4 proteins (2^2) of differing composition and/or length. Splicing aberrations occur in helical element IVS3 and neighboring interhelical loops. All four variants are not detected for each group. Splicing variations within helical element IVS3 have also been reported for class rbC brain channels (102).

Subcellular distribution of L-type Ca^{2+} channels is believed to be postsynaptic (105,106). Recent immunocytochemical studies, using monoclonal bodies raised against neuronal $\alpha_2\delta$ subunits (MANC1 &3), localize L-type channels to cell soma and proximal dendrites (94,95). A high density of channels was also seen at the trunk of major dendritic trees. The functional significance of L-type Ca^{2+} channel distribution is of current and critical importance (33,95).

ω-CgTx Receptors: N-type Voltage-dependent Ca^{2+} Channels - A 27 amino acid peptide ω-CgTx GVIA (fig 3), isolated from the fish-eating marine snail *Conus geographus* (107-110), persistently inhibits N (25,111) and L-type (25) neuronal Ca^{2+} channels. Specific high-affinity toxin binding sites have been characterized in a number of neuronal membrane preparations (112-121) and neuronal cell lines (133,134). In regional binding studies in rat brain, [^{125}I-Tyr^{22}]-ω-CgTx binding sites (B_{max}) were 10-80 times more abundant than [^{3}H]-nitrendipine binding sites (121). Cross-linking of radioactive derivatives of ω-CgTx employing chemical (113,116,118) and photoaffinity (114-116,119,120) reagents has been employed to describe the peptides associated with the ω-CgTx receptor in intact synaptosomal membranes from chick (112,116), bovine (115) and rat brain (114,118-120). Chemical cross linking of [^{125}I]-ω-CgTx to receptors found in chick synaptosomes with disuccinyl suberate identified a protein with a native mass of ~170kDa (112,116) which after reduction migrates with an apparent mass of ~140kDa (112). The electrophoretic behavior of this protein under native and reducing conditions closely resembles that of the $\alpha_2\delta$ subunit from skm DHP-receptor (vide supra). A similar study in rat brain (118) marked a peptide of ~300 kDa (under reducing conditions). However, photoaffinity labelling of synaptosomal receptors with ANB-[^{125}I]-ω-CgTx (ANB: 4-azido-2-nitro-benzoyl) identified peptides of ~220 kDa from chick (116), and of ~310 kDa, ~230 kDa and ~ 34 kDa from bovine or rat brain (114,115,119,120). Apparently, the tissue type and toxin-based affinity reagent used determines the protein(s) identified. The peptide composition of the ω-CgTx receptor and its relationship to the N-type Ca^{2+} channel remains unclear and new toxin ligands, affinity probes or immunological reagents (122) may be required to selectively identify proteins associated with the N-type Ca^{2+} channel (vide infra).

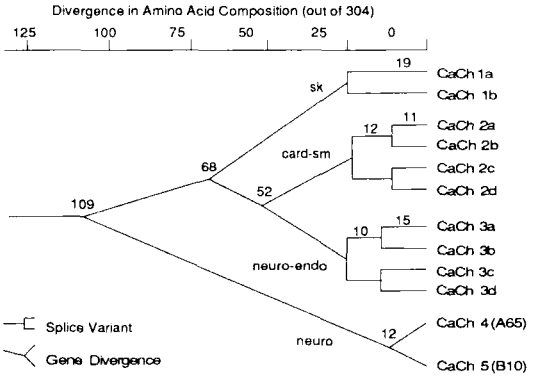


Figure 5. Diversity of deduced aminoacid sequence of cloned α_1 subunits from L-type Ca^{2+} channel genes. Adapted with permission from E. Perez-Reyes, et al. (ref. 98).

Voltage-dependent Ca^{2+} Channel Toxins - Because of their low lipid solubility and high-affinity selective-binding interactions, toxins are recognized as useful tools for classifying and studying voltage and receptor-gated ion channels (108,109,123-128). The well-known ω-conotoxins (eg. ω-CgTx GVIA, ω-CgTx MVIIA) have been used extensively as experimental tools for characterizing neuronal Ca^{2+} channels (vide supra,108,109,123,129,130). Recent work shows that spider venom contains a rich assortment of unique pharmacological tools (131,132) including selective neuronal Ca^{2+} channel ligands (135-148). Such potential was first suggested by reports that unidentified peptide toxins present in the venom of *Plectreurys tristes* (117) and the funnel-web spider *Agelenopsis aperta* (149) inhibit ω-CgTx binding to rat brain membranes suggesting an action on voltage-sensitive Ca^{2+} channels. Two toxins isolated from *Agelenopsis aperta* , ω-Aga-I A (~7.5kDa) and ω-Aga- I I I A (~8.5kDa), are potent blockers of N and L-type voltage-sensitive Ca^{2+} channels in rat dorsal root ganglion, hippocampal and cerebellar neurons (138,148) with an effective concentration range of 10-100 nM. The ω-agatoxins are distinguished given ω-Aga-I I I A potently blocks ω-CgTx binding to chick synaptosomal membranes (EC_{50}=10-20 nM) and inhibits depolarization induced $^{45}Ca^{2+}$ uptake into chick brain synaptosomes (EC_{50}=1-5nM) while ω-aga- I A is inactive (140,146,150). While no sequence data are available for ω-Aga- I I I A , a primary sequence (66 aa) has been proposed for ω-Aga- I A (140).

A low molecular weight (200-400 Da) constituent produced by *Agelenopsis aperta* , termed FTX, was shown to selectively block a Ca^{2+} channel found in cerebellar Purkinje cells. This channel, though blocked by Cd^{2+} and Co^{2+} (0.5 μM), was refractory to ω-CgTx and dihydropyridines. An affinity gel, constructed by immobilization of FTX on Sepharose 4B, was used to isolate the Purkinje cell channel. Single channel recordings from channels reconstituted in black lipid bilayers display unique biophysical properties (conductance (80 mM Ba^{2+}) 10-12 ps, activation potentials at > -60mV). From these data it was concluded that FTX acts on a new type of Ca^{2+} channel (141) termed the P channel (for Purkinje). Rat brain mRNA injected *Xenopus* oocytes express a Ca^{2+} current, which activates at -40mV, that is totally insensitive to ω-CgTx and the dihydropyridines (74,142). This current is partially blocked by FTX (~60%) and therefore is biophysically and pharmacologically similar to the P channel (142). Unfortunately, no rigorous structural data on FTX has been made available (143). These toxins or related materials will prove to be valuable tools in studies of neuronal Ca^{2+} channel isolation and characterization which will invariably lead to a better understanding of subtype diversity, molecular structure and function.

Conclusions - One clear conclusion from the physiological and structural studies cited herein is that there is a multiplicity of Ca^{2+} channel types in neurons. A correspondence between the diversity in channels based on biophysical criteria such as inactivation rate and pharmacological criteria such as sensitivity to ω-CgTx and specific structural types is not yet possible. However, the molecular biology of neuronal Ca^{2+} channels is a rapidly developing research area and it will soon be possible to test specific hypotheses regarding the properties of structurally defined channels.

The multiplicity of channels is an imperfect fit with the T,L,N scheme that has been used in the literature and that we used here. However, this scheme remains useful until a molecular pharmacology of neuronal Ca^{2+} channels has been described or until new, highly specific, pharmacological agents are developed.

There are multiple species of neuronal Ca^{2+} channels that cannot be encompassed in the familiar muscle L-channel (DHP-sensitive) class. One implication of the recognition of these non-L channels is that therapeutic Ca^{2+} channel blockers currently in use (DHP, etc.) do not act on large populations of neuronal Ca^{2+} channels; thus, there are no clinical data relevant to the effects of modulating these channels in human disease.

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Chapter 5. Recent Advances In Neurokinin Receptor Antagonists

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Introduction - The neurokinins, a class of peptide neurotransmitters characterized by the common C-terminal sequence Phe-X-Gly-Leu Met-NH_2, are widely distributed in the central and peripheral nervous systems. Although the neurokinins have been the object of research for well over 50 years, progress in delineating their exact role and function has been slow. This is due, in large part, to the absence of potent and selective neurokinin antagonists. In this chapter, the most prominent members of the neurokinin family and their receptors are introduced, followed by a brief description of their physiological roles, potential therapeutic applications of neurokinin antagonists, and some of the pharmacological tools used to study the neurokinins. Also included is a review of neurokinin antagonists and their pharmacology, with particular emphasis placed on the more recently reported potent and selective antagonists.

The Neurokinins - The first member of the neurokinin family to be reported was substance P (SP), isolated in 1931 by von Euler and Gaddum (1). In 1971, Leeman and Chang determined the amino acid sequence of substance P and identified it as an undecapeptide (2). In 1983, two additional peptides sharing the same C-terminal sequence were demonstrated to be present in mammalian tissue (3-5). Although initially called by several different names, they are now identified as neurokinin A (NKA) and neurokinin B (NKB).

Substance P (SP)	Arg-Pro-Lys-Pro-Gln-Gln-**PHE**-Phe-**GLY-LEU-MET-NH_2**
Neurokinin A (NKA)	His-Lys-Thr-Asp-Ser-**PHE**-Val-**GLY-LEU-MET-NH_2**
Neurokinin B (NKB)	Asp-Met-His-Asp-Phe-**PHE**-Val-**GLY-LEU-MET-NH_2**

Neurokinin precursors and their mRNA's have been reviewed recently (6). There are three mRNA's that encode for substance P, which are denoted α-, β-, and γ-preprotachykinin (PPT) mRNA. α-PPT contains only the SP peptide sequence. β-PPT contains SP and NKA (7) - as well as neuropeptide K (NPK), an N-terminally extended form of NKA (8). Similarly, γ-PPT contains SP, NKA, and neuropeptide γ (NPγ), an N-terminally extended form of NKA (9). Thus, SP and NKA (as well as NPK and NPγ under some circumstances) are often co-synthesized and co-released. NKB is encoded by an entirely different PPT gene (10,11).

Neurokinin Receptors - There are at least three known receptors for the three neurokinins, designated NK-1, NK-2, and NK-3 (12). The endogenous ligands for these receptors are believed to be SP, NKA, and NKB, respectively, based on relative potency.

NK-1: SP>NKA>NKB
NK-2: NKA>NKB>SP
NK-3: NKB>NKA>SP

All three of the receptors were cloned recently. The bovine NK-2 receptor was the first to be cloned, and was found to be a member of the G-protein coupled receptor superfamily (13). These receptors are characterized by seven α-helical transmembrane spanning regions, with an extracellular amino terminus and a cytoplasmic carboxy terminus. The rat (14) and human (15,16) NK-2 receptors have been cloned more recently. The receptors from the three species show approximately 85% sequence identity to each other when compared pairwise. The rat NK-1 and NK-3 receptor sequences are now known as well (17-19) and show a significant amount of sequence homology to the NK-2 receptor. In the portions of the rat NK-1, NK-2, and NK-3 receptors containing the seven transmembrane regions and their connecting loops, there is 54% homology between NK-1 and NK-2, 66% between NK-1 and NK-3, and 55% between NK-2 and NK-3 (19). The NK-1 (20-23), NK-2 (22,24), and NK-3 (25) receptors have all been shown to stimulate inositol phospholipid hydrolysis in their signal transduction pathways.

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The distribution of the neurokinin receptors within the body has been studied using several experimental techniques, including quantitative autoradiography and RNA blot hybridization analysis. Although the results are not completely consistent across studies, the NK-1 receptor is clearly located in both the central and peripheral nervous systems (26-29), the NK-2 receptor is located primarily in the peripheral nervous system (28-30), and the NK-3 receptor is located primarily in the central nervous system (26-29). It should be noted that the bulk of this work has been done in rats, and it is not known how well it would translate to other species. It is also clear that in many locations the density of a particular neurokinin does not match that of its receptor.

Biological Actions - Although SP was discovered well over 50 years ago, there is not yet a clear understanding of the role neurokinins play in normal physiology and in disease states, particularly in humans. They appear to be implicated in pain and inflammation, the immunological response, and in central nervous system (CNS) function.

The importance of the neurokinins has been most clearly demonstrated in pain and inflammation. SP and NKA are localized in C-afferent sensory neurons. Upon depolarization of these neurons, SP and NKA are released in the dorsal horn of the spinal cord and in the periphery via an axon reflex (31-32). In the spinal cord, it is believed that they transmit information that a painful stimulus has occurred (33-35). In support of this, intrathecally administered SP, NKA, and NK-1 selective agonists are hyperalgesic (36). In contrast, NKB has an analgesic effect that appears to be mediated through spinal release of an opioid (36).

In the periphery, release of SP and NKA leads to an inflammatory response, including vasodilatation, plasma extravasation, and mobilization of cells of the immune system (37,38). Some disease states in which the evidence for the involvement of neurokinins has been explored most extensively are arthritis (39,40), inflammatory bowel disease (SP) (41-43), and asthma and related problems (SP and NKA) (44-46). Thus, it is believed that a neurokinin antagonist is potentially useful as a non-narcotic analgesic and an anti-inflammatory agent.

The CNS effects of the neurokinins are less clear than those in the periphery and were reviewed recently (47). One of the best characterized effects is the interaction of SP and NKA with the dopaminergic system. There is evidence that both SP and NKA activate the dopaminergic pathway (possibly by different mechanisms) (48), and that dopamine receptor antagonists downregulate the synthesis of SP and NKA (49). This has led to speculation that an SP agonist would be useful in Parkinson's disease, which is characterized by the degeneration of substantia nigral dopamine neurons (50).

There is also evidence that the neurokinins interact with other central monoamine systems, such as serotonin and acetylcholine (47). In addition, recent evidence indicates that SP acts as a neurotrophic factor for differentiating hippocampal neurons. This could have implications in the pathology of Alzheimer's disease (51). Although much work has been done to gain an understanding of the CNS effects of the neurokinins, the therapeutic potential of selective agonists or antagonists is still far from being understood.

Pharmacology - One of the major advances in this field in the past several years has been the development of better pharmacological tools to aid in the study of the neurokinins. In earlier work, tissue preparations containing more than one receptor subtype and agonists that acted at more than one receptor subtype were used in the evaluation of potential antagonists. There are now fairly selective tissue preparations and selective, relatively potent agonists. Some of the more commonly accepted agonists and models are shown in Table 1. Atropine-blocked guinea pig ileum (GPI) is still fairly commonly used for NK-1, although it contains several receptor subtypes (52).

In addition to the selective bioassays, there are also receptor binding assays for NK-1, NK-2, and NK-3 receptors. The following radiolabelled ligands are commercially available and frequently used: ^{125}I-Bolton-Hunter SP and ^{3}H-SP for NK-1; ^{125}I-histidyl NKA for NK-2; and ^{125}I-Bolton-Hunter eledoisin (ELE, an amphibian tachykinin) for NK-3. More recently, radiolabelled forms of the more selective neurokinin agonists have also been described: ^{3}H-[Sar^{9},Met(O$_2$)^{11}]SP(6-11) for NK-1; ^{125}I-NPγ for NK-2; and ^{3}H-senktide for NK-3 (30,53).

Table 1. Selective Neurokinin Agonists and Tissue Preparations

Receptor	Endogenous Ligand	Selective Agonists	Tissue Preparations
NK-1	Substance P	[Sar^9,Met(O$_2$)^{11}]SP(6-11) (54) [pGlu^6,Pro^9]SP(6-11) (Septide) (57) SP-OMe (58)	Rabbit Jugular Vein (RJV) (55) Dog Carotid Artery (DCA) (56)
NK-2	Neurokinin A Neuropeptide K Neuropeptide γ	[Nle^{10}]NKA(4-10) (53) [β-Ala^8]NKA(4-10) (59)	Rabbit Pulmonary Artery (RPA) (56) Hamster Trachea (HT) (60) Rat Vas Deferens (RVD) (61)
NK-3	Neurokinin B	[MePhe^7]NKB (53) Succ-[Asp^6,MePhe^8]SP(6-11) (Senktide) (62)	Rat Portal Vein (RPV) (56)

NEUROKININ ANTAGONISTS

The search for potent substance P antagonists began well before the complex nature of the neurokinin family and its receptors was understood. Not surprisingly, many of the earlier antagonists lack receptor subtype specificity. On the other hand, these compounds laid the groundwork for much of the more recent research in which more potent and selective compounds have been developed, and they have served as pharmacological tools for increasing our understanding of the neurokinins and their actions. First, the "general" neurokinin antagonists will be described, followed by compounds having specificity for the NK-1, NK-2, and NK-3 receptor subtypes.

General Neurokinin Antagonists - This group of antagonists consists of compounds lacking specificity and compounds whose specificity is unknown. The assay most commonly used to evaluate these antagonists is inhibition of SP-induced contractions in guinea pig ileum. In some cases, because of interest in the involvement of SP in pain transmission, the compounds were evaluated in some of the traditional analgesia assays.

A selection of neurokinin antagonists having modified SP sequences and their activity in guinea pig ileum are shown in Table 2. The activity is given as the pA_2, the negative logarithm of the antagonist concentration at which twice the agonist concentration is required to achieve the response obtained in the absence of the antagonist. The majority of these compounds were reviewed in 1986 (63), and a set of generalizations were made based on the compounds reviewed at that time. The more recently prepared compounds do not change these generalizations. In order to show antagonist activity, the minimum sequence required is SP(7-11). However, antagonist potency is enhanced in the compounds possessing more of the C-terminal portion of SP. The most potent compounds ($pA_2 > 8.0$) are modifications of the full SP(1-11) sequence, but compounds of reasonable potency ($pA_2 > 7$) can be prepared based on SP(4-11). Compare, for example, **1** ($pA_2 = 8.1$) and **4** ($pA_2 = 7.1$). The structural feature conferring antagonism on all of these compounds is the replacement of Phe^7 and Gly^9 by D-Trp or another aromatic D-amino acid (63), with other alterations typically made in the N-terminal portion. In the case of the undecapeptides, the positively charged Arg^1 and Lys^3 have been implicated in the histamine-releasing properties of SP (64), so alteration of these residues is desirable. Substitutions of D- or L-Arg or Pro are also commonly made in the N-terminal portion of these peptide antagonists to improve metabolic stability and/or improve solubility (63). At the C-terminus, replacement of Met^{11} by Nle, Leu, or Phe improves potency in many, but not all, cases (63). For example, compare **7**, **8**, and **9**.

Compounds such as those in Table 2 have been used to test the hypothesis that an SP antagonist should exhibit analgesic properties. Most of these data were reviewed in 1988 (65). On the basis of the available data, the hypothesis cannot be unequivocally validated. The relatively low selectivity and potency of these compounds are probably important factors. It was also found that some of the antagonists tested in pain assays in rat spinal cord led to flaccid paralysis (65). Inspection of the spinal cords showed a significant amount of necrosis, possibly due to ischemia

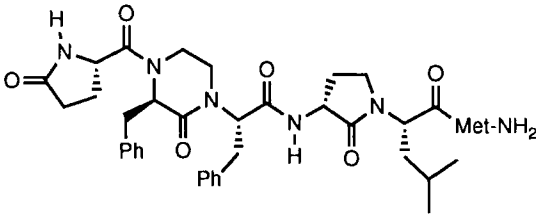
(66). Toxicity appears to be less problematic in the mouse (67,68). Finally, there are cases in which the results obtained in analgesia assays in different laboratories are inconsistent with one another, perhaps due to differences in methodology or to species differences (65).

Table 2. General Neurokinin Antagonists

	Antagonist	pA_2, Guinea Pig Ileum	Ref.
Undecapeptides			
1	[D-Lys(Nic)^1,Pal(3)^3,D-Phe(Cl_2)^5,Asn^6,D-Trp^{7,9},Nle^{11}]SP (Spantide II)	8.10	69
2	[D-Arg^1,Nle^3,D-Phe(Cl_2)^5,Asn^6, D-Trp^{7,9},Nle^{11}]SP	7.70	70
3	[D-Arg^1,D-Trp^{7,9},Leu^{11}]SP (Spantide)	7.10	71
Octapeptides			
4	[D-Pro^4,D-Trp^{7,9},Nle^{11}]SP(4-11)	7.10	72
5	[D-Pro^4,D-Trp^{7,9,10},Phe^{11}]SP(4-11)	7.02	73
6	[D-Pro^4,D-Trp^{7,9,10}]SP(4-11)	6.60	73
Heptapeptides			
7	[Arg^5,D-Trp^{7,9},Nle^{11}]SP(5-11)	6.71	74
8	[Arg^5,D-Trp^{7,9}]SP(5-11)	5.91	74
9	[Arg^5,D-Trp^{7,9},Phe^{11}]SP(5-11)	5.70	74
Hexapeptides			
10	[Pro^6,D-Trp^{7,9},MeLeu^{10}]SP(6-11)	6.30	75
11	[Pro^6,D-Trp^{7,9}]SP(6-11)	5.70	75
Pentapeptides			
12	[D-Trp^{7,9},Nle^{11}]SP(7-11)	5.31	76

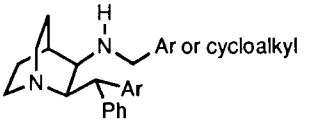
There are several series of compounds for which binding and tissue bath data are not available, but which bear mentioning. In 1981, a patent was issued describing a series of compounds based on SP(6-11) containing an ethylene bridge linking the peptide backbone nitrogens of Phe^7 and Phe^8 (77). The compounds were claimed as analgesics and psychotherapeutic agents rather than SP antagonists, but subsequently published data demonstrate that, when administered intraspinally to mice, some of the compounds inhibit SP and capsaicin-induced scratching and are analgesic in the hot plate test (78). No negative effect on motor behavior was observed for these compounds. Additional support for the neurokinin antagonism of at least some members of this class comes from work in other laboratories, in which U-67,202 (13) is described as a selective NK-2 antagonist (79).

13

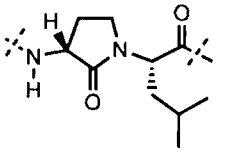


A patent covering a series of 1,2-cis-disubstituted quinuclidines as substance P antagonists was issued recently (80). These compounds are represented by 14. No receptor binding or tissue bath data were provided (81).

14



NK-1 Selective Antagonists -- L-668,169 (**15**), a compound showing good selectivity for the NK-1 receptor subtype, is a cyclic dimer of a modified SP(6-11) sequence and has conformational restrictions in the backbone as a result of the ANC-2 group (82). Compound **15** has a pA_2 of 7.0 in an NK-1 assay (atropine-blocked guinea pig ileum, eledoisin as agonist); 5.1 in an NK-2 assay (rat vas deferens, eledoisin); and 5.6 in an NK-3 assay (rat portal vein, eledoisin). Receptor binding data were not given.

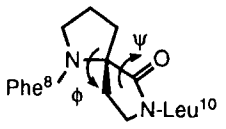


-R-Gly[ANC-2]-Leu-

cyclo(Gln-D-Trp-(N-Me)Phe-R-Gly[ANC-2]-Leu-Met)$_2$

15

The successful design of NK-1 selective antagonist GR71251 (**16**) using a somewhat different approach was described recently (83). This method consisted of the use of molecular modelling and conformational energy maps of ϕ and ψ in Gly^9 in NK-1 and NK-2 agonists having some conformational constraints, in order to determine families of low energy conformations. From these studies, a conformationally rigid spirolactam was designed in which these agonist conformations could not be attained. Then, structure-activity relationships were used to optimize antagonist potency at the NK-1 receptor. Compound **16** is one of the most potent and selective peptide NK-1 antagonists known. It has a pA_2 of 7.76 in an NK-1 assay (atropine-blocked guinea pig ileum, SP methyl ester as agonist). Based on comparisons of dose ratios, the antagonist selectivity is approximately 1,000 relative to the NK-2 (rat colon muscularis mucosae) and NK-3 receptors (rat portal vein). No agonism was apparent at any of the receptors at concentrations up to 30 uM.



Phe^8-SPIRO-Leu^{10}

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-SPIRO-Leu-Met-NH_2

16

NK-2 Selective Antagonists - In 1988, two antagonists derived from NKA(5-10) showing good selectivity for the NK-2 receptor were described (82). The most potent and selective was L-659,877 (**17**), a cyclic hexapeptide containing only L-amino acids. It has a pA_2 of 5.9 in an NK-1 assay (atropine-blocked guinea pig ileum, eledoisin as agonist); 8.0 in an NK-2 assay (rat vas deferens, eledoisin); and 5.9 in an NK-3 assay (rat portal vein, eledoisin). Interestingly, L-659,874 (**18**), which is an open chain version in which the equivalent of the Gly^8-Leu^9 peptide bond has been cleaved, is also NK-2 selective, although somewhat less potent: $pA_2 < 4.5$ at NK-1; 6.8 at NK-2; and 4.4 at NK-3. Both **17** and **18** inhibited the binding of ^{125}I-Bolton-Hunter eledoisin to hamster urinary bladder, an NK-2 preparation, with calculated IC_{50}'s of 11 and 44 nM, respectively (84). The NK-2 antagonist activity and selectivity of **18** was additionally confirmed by its ability to inhibit eledoisin-induced inositol phospholipid hydrolysis in hamster urinary bladder but not rat parotid gland (an NK-1 preparation) (85). Compounds **17** and **18** were also among the first to provide evidence for the possibility of an additional neurokinin receptor or receptor subtype. Both were significantly less potent in guinea pig trachea (believed to be a tissue in which the NK-2 receptor is the predominant one) than in rat vas deferens (82).

17 cyclo(Gln-Trp-Phe-Gly-Leu-Met)

18 Ac-Leu-Met-Gln-Trp-Phe-Gly-NH_2

Several NK-2 selective antagonists have pA_2 values that are not consistent across different "NK-2 selective" tissue bath assays, although a series of reference agonists show similar activity in these assays. MEN 10,207 (**19**) and MEN 10,208 (**20**) are based on NKA(4-10) and NKA(3-10), respectively, and possess D-Trp at positions 6, 8, and 9 to confer competitive antagonism (86). Tyr in position 5 and Arg in position 10 increased the affinity and NK-2 selectivity of these compounds. R 396 (**21**) is a modification of **18** in which the Met residue has been replaced with Asp. Both **19**

and **20** show much more potent antagonism in the rabbit pulmonary artery (RPA) than in the hamster trachea (HT), while **21** shows just the opposite effect (Table 3). Other variables were eliminated as causes for the different pA_2's, leaving the strong possibility that receptor subtypes exist.

Table 3. NK-2 Antagonists: Tissue Bath Data

	Antagonist	pA_2-RPA	pA_2-HT
19	Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2	7.65	5.80
20	Thr-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2	7.47	5.93
21	Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2	5.42	7.67

Similarly, receptor binding data on several NK-2 selective antagonists in SKLKB82#3 cells (a murine fibroblast cell line transfected with bovine stomach cDNA) and hamster urinary bladder (HUB) reveal differences between these two preparations (Table 4) (87). MDL-29,913 (**22**) is a modification of **17** in which Leu has been substituted for Met^{10}, and the Leu^9-Leu^{10} amide bond has been reduced to the N-methyl methyleneamino moiety. Like **18**, **22** inhibits PI turnover in HUB (pA_2 = 8.2). Compounds **22**, **17**, and **18** are 22-30 times less potent in the bovine-derived assay than in HUB. Conversely, **19** is approximately 100 times more potent in the bovine-derived assay.

Table 4. NK-2 Antagonists: Receptor Binding Data

		K_i (nM)	
	Antagonist	SKLKB82#3	HUB
22	cyclo[Leu(ψCH_2NCH_3)Leu-Gln-Trp-Phe-Gly]	20	0.75
17	cyclo(Gln-Trp-Phe-Gly-Leu-Met)	38	1.75
18	Ac-Leu-Met-Gln-Trp-Phe-Gly-NH_2	308	10.5
19	Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2	15	1796

Based on these data, it appears likely that **17** and **18** and compounds derived from them show higher affinity for the NK-2 receptor found in hamster urinary bladder and hamster trachea. Conversely, NK-2 selective antagonists such as **19** and **20**, prepared using the traditional approach of D-amino acid substitution, show higher affinity for the rabbit and bovine receptors.

NK-3 Selective Antagonists - The first NK-3 selective antagonist was prepared following the approach of aromatic D-amino acid substitution, particularly in the C-terminal portion of NKB. The resulting compound, [D-Pro^2,D-$Trp^{6,8}$,Nle^{10}]-NKB (**23**), was tested for its ability to inhibit contractions produced by SP, NKA, and NKB in guinea pig ileum. Competitive inhibition was observed only for NKB (pA_2 = 5.5) (88-90). With SP and NKA as agonists, approximately 20% non-competitive inhibition was observed at concentrations at which the NKB contractions were inhibited (90). Compound **23** also antagonized reciprocal hind-limb scratching induced by intrathecal NKB injection, while having no effect on scratching induced by SP or NKA (89).

A somewhat different approach led to a series of NK-3 selective antagonists based on NKB(3-10) that are slightly more potent than **23**. It was known that the N-terminal dipeptide is not necessary for contractile activity (91). It was subsequently found that substitution of Phe^6 by Gly or D-Ala led to compounds having greatly reduced contractile activity as well as (in some cases) antagonist activity (92). Thus, the compounds in Table 5 antagonized the action of NKB but not SP or NKA on guinea pig ileum (GPI).

Table 5. NK-3 Antagonists

	Antagonist	pA_2 (GPI)
24	[Gly^6]NKB(3-10)	5.82
25	[Arg^3,D-Ala^6]NKB(3-10)	5.69
26	[Arg^3,Gly^6,D-Trp^8]NKB(3-10)	6.30

A series of more potent NK-3 antagonists based on NKA(4-10) and NKB(4-10) was recently described (93). These compounds are characterized by the replacement of Gly^8 with β-Ala, and of Val^7 by an aromatic amino acid, such as Tyr, Phe, MePhe, or Trp. The two most potent antagonists, [Trp^7,β-Ala^8]NKA(4-10) (**27**) and [$MePhe^7$,β-Ala^8]NKB(4-10) (**28**), have pA_2's greater than 7 in an NK-3 selective preparation (rat portal vein, [$MePhe^7$]NKB as agonist) (Table 6). They exhibit no antagonism in NK-1 or NK-2 preparations (NK-1: dog carotid artery or rabbit jugular vein, Ac[Arg^6,Sar^9,$Met(O_2)^{11}$]SP(6-11) as agonist; NK-2: rabbit pulmonary artery, [Nle^{10}]NKA(4-10) as agonist). Compounds **27** and **28** do show some agonism at the NK-1, NK-2, and NK-3 receptors at somewhat higher concentrations, which poses a problem. It is interesting that 1) these compounds contain no D-amino acid substitutions and 2) some of the compounds described (including the most potent antagonist) are based on NKA rather than NKB.

Table 6. More Potent NK-3 Antagonists

	Antagonist	pA_2 (RPV)
27	[Trp^7,β-Ala^8]NKA(4-10)	7.46
28	[$MePhe^7$,β-Ala^8]NKB(4-10)	7.26

Conclusion - The development of potent and selective NK-1, NK-2, and NK-3 antagonists has been steadily progressing over the past five years. At this time, there are selective antagonists having pA_2's of greater than 7 for each of the three known neurokinin receptors. At the same time, these compounds are raising the strong possibility that there are additional NK receptors, receptor subtypes, species differences, or a combination of the three. The recently described non-peptide quinuclidine neurokinin antagonists could become important tools, should the compounds be found to be potent and selective. It is hoped that these peptide and non-peptide antagonists will further the efforts to understand the biological actions of the neurokinins in both normal and disease states as well as the potential therapeutic utility of neurokinin antagonists and agonists.

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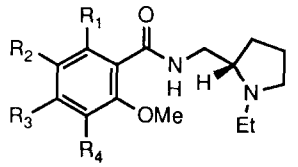
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Chapter 6. Antipsychotics

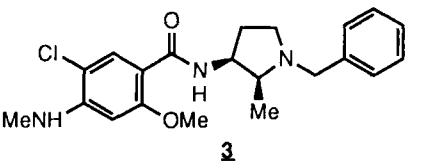
Lawrence D. Wise and Thomas G. Heffner
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Introduction - Antipsychotic drugs are used primarily for the treatment of schizophrenia but also have more limited applications in mania, schizoaffective disorder, anxiety, dementia and drug overdose. Although available antipsychotic drugs typically reduce delusions and hallucinations (1), symptoms of schizophrenia such as poverty of speech and social withdrawal are less improved (2). In addition, some patients obtain little benefit from these drugs (1) and relapse occurs frequently (3). Available antipsychotics also produce serious neurological side effects, including extrapyramidal syndrome (EPS) and tardive dyskinesia (4). These limitations have stimulated efforts to identify new drugs with improved therapeutic profiles. The history (5), clinical use (6,7,8) and pharmacokinetics (9) of antipsychotics have been recently reviewed.

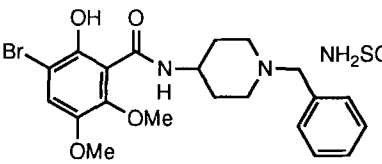
Dopamine (DA) D_2 Receptor Antagonists - Both the efficacy and neurological side effects of available antipsychotics have been attributed to their blockade of brain DA receptors. The well-known correlation between clinical potency and affinity for DA D_2 receptors has supported continued research on DA D_2-selective benzamides. A series of articles on remoxipride (1) described both its preclinical and clinical profiles (10-12). In preclinical studies, 1 was found to have affinity for DA D_2 receptors and sigma binding sites in brain, but to lack appreciable affinity for DA D_1, serotonin (5HT), noradrenaline, acetylcholine and histamine receptors (11). Reports of clinical trial results indicated that 1 provided efficacy comparable to that of haloperidol with a lower incidence and intensity of neurological side effects (12). However, some patients given 1 required medication for EPS, suggesting that the drug was not free of EPS liability (13). Both amisulpride (2) and emonapride (YM-09151, 3) were also reported to have efficacy with less EPS than haloperidol in clinical trials (14,15).



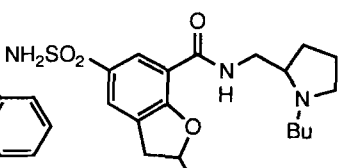
	R^1	R^2	R^3	R^4
1	OMe	H	H	Br
2	H	H	NH_2	SO_2Et
4	OH	Br	H	OMe
5	H	Br	H	OMe



3



6



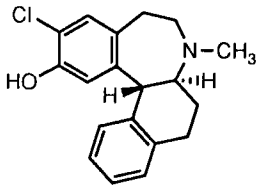
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The SAR of benzamide derivatives continued to be the subject of preclinical studies. Both the 5,6-dimethoxysalicylamides, e.g., FLB 463 (4), and their 2-deshydroxy analogs, e.g., FLB 457 (5), were found to be potent and stereoselective DA D_2 receptor antagonists (16-18). While the lipophilicity of the 3-substituent appears to be of major importance for the 6-methoxysalicylamides, DA D_2 receptor affinity of these series was only marginally influenced by the nature of the additional ring substituent (18,19). Like the 4-piperidinylbenzamides, the related salicylamides

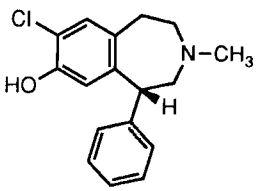
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require a lipophilic N-substituent such as a benzyl group for high DA D_2 affinity (20). QSAR studies on a series of 4-piperidinyl derivatives led to the synthesis of the 6-methoxysalicylamide **6** with enhanced potency for DA D_2 receptors. Evaluation of a series of benzamides in which the 2-methoxy group was mimicked by a dihydrobenzofuran ring, stabilizing the intramolecular hydrogen bonding, resulted in identification of **7**, a compound with potent antipsychotic-like activity (21). A model of the pharmacophore for benzamides was developed using computer modeling and extended to account for structural features of other DA D_2 antagonists (22).

DA D_1 Receptor Antagonists - Blockade of DA D_1 receptors has been proposed to play an important role in the action of certain antipsychotics including clozapine (23). Studies with SCH 39166 (**8**), a compound with high affinity and selectivity for DA D_1 receptors, revealed a unique distribution of cortical binding sites relative to the prototypal DA D_1 antagonist, SCH 23390 (**9**, 24). Chronic administration of **8** to rats and rhesus monkeys increased the number of DA D_1, but not DA D_2 receptors, demonstrating its *in vivo* selectivity (25). The profiles of **8** and **9** in pharmacological tests predictive of antipsychotic efficacy appear to be similar, with **8** having a longer duration of action (26).



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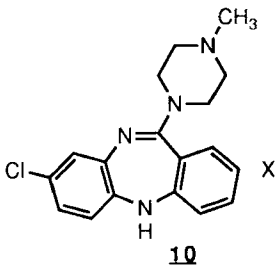


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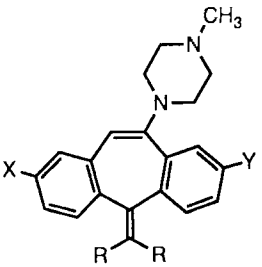
The biological conformations of a series of benzazepine DA D_1 agonists and antagonists were estimated from molecular mechanics calculations (27). Their most probable receptor-bound orientation was determined to be a chair conformation with an equatorial phenyl group and, in the case of **9**, an equatorial N-methyl substituent. Chronic treatment with **9** decreased DA metabolism in both the striatum and mesolimbic area in rats, effects seen also with haloperidol (28). This as well as conflicting reports on the effects of DA D_1 antagonists in primate models of antipsychotic-induced EPS (29) raise questions about the EPS liability DA D_1 antagonists.

Multiple Receptor Antagonists - Reviews underscored the improved antipsychotic efficacy profile of clozapine (**10**) relative to other antipsychotics, including benefit for treatment-resistant schizophrenics and improvement in quality of life (30-32). Such findings have stimulated further proposals about mechanisms responsible for the unique activity of **10** (33,34), including cortical DA effects (35,36), effects on DA D_2, DA D_1, $5HT_2$ and cholinergic receptors (37-39) and effects on neuropeptide systems (40,41).

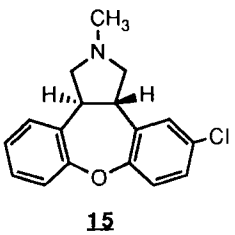
A number of new compounds have been proposed as possible clozapine-like agents. A report on compounds related structurally to clozapine revealed that **11**, **12** and **13** had affinity for DA D_1, DA D_2 and muscarinic receptors while **14** retained affinity only for DA D_1 and muscarinic sites (42).



10



	R	X	Y
11	H	Cl	H
12	H	H	Cl
13	CH_3	Cl	H
14	CH_3	H	Cl

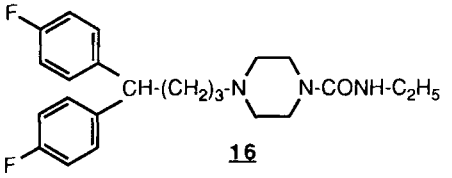


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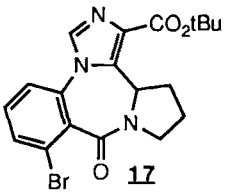
The preclinical activity of ORG-5222 (**15**) was profiled in a series of papers (43-45). Like clozapine, **15** was found to have high affinity for DA D_1, DA D_2, alpha$_1$ adrenergic, alpha$_2$ adrenergic, $5HT_2$, and histamine H_1 receptors (but not muscarinic receptors) and was active in behavioral tests for DA D_2 antagonist effects.

Serotonergic Agents - The ability of clozapine to block $5HT_2$ and DA receptors, as well as known interactions between DA and 5HT neural systems in brain, has renewed interest in the role of brain 5HT in antipsychotic drug action. One proposal has suggested that the unique profile of clozapine stems from its balanced blockade of brain DA and 5HT receptors (46,47), a theory that appears to be supported by recent preclinical studies (48,49). A variety of compounds with $5HT_2$ antagonist effects combined with varying degrees of DA antagonist effects are in development.

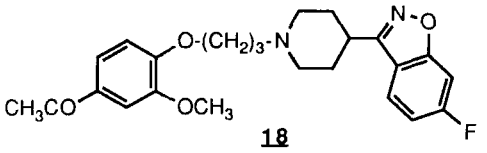
Amperozide (**16**) was among compounds that appear to have more prominent effects on brain 5HT than on DA receptors, as described in a series of preclinical studies (50). Compound **16** had high affinity for $5HT_2$ receptors, modest affinity for alpha$_1$ adrenergic receptors and relatively low affinity for DA D_2, DA D_1, $5HT_{1A}$ and other receptors (51). It was reported to show preclinical antipsychotic-like activity (52- 54), to increase DA release from both striatal and limbic neurons (55), to reduce amphetamine-stimulated DA release and to decrease neurotransmitter uptake (56). Unlike most DA antagonists, **16** decreased plasma prolactin levels in rats (57) and stimulated tuberoinfundibular DA neurons, an effect seen also with clozapine (58). In addition, **16** was active in preclinical tests predictive of anxiolytic and antidepressant activity (52). ICI 204,636 (**17**), also appears to be a potent $5HT_2$ antagonist with weak DA D_2 antagonist activity (59). While active in behavioral tests predictive of antipsychotic efficacy, **17** did not produce EPS in monkeys (60,61). HP 873 (**18**) was found to have high affinity for $5HT_2$ and alpha$_1$ adrenergic receptors but weak affinity for DA D_2 receptors and was active in preclinical antipsychotic tests (62-64).



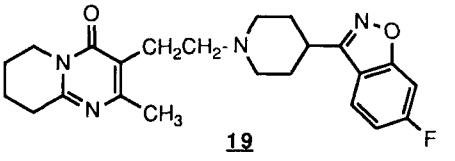
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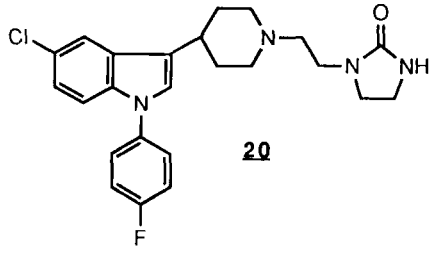


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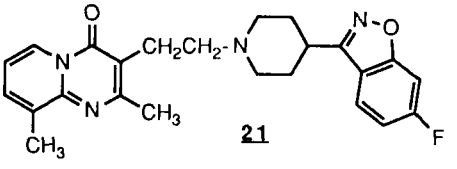


19

Other compounds combine potent blockade of 5HT and DA receptors. Risperidone (**19**), a drug with high affinity for $5HT_2$ and DA D_2 receptors, was reported to improve negative schizophrenic symptoms and to cause a low incidence of EPS in open-label clinical trials (65-67). Sertindole (**20**), a compound with both $5HT_2$ and DA D_2 affinity, blocked effects of LSD and quipazine but not DA agonists (68). Chronic dosing with **20** decreased the number of spontaneously firing brain DA neurons, an effect seen with DA D_2 antagonists (69). The substituted pyridopyrimidinone, R-79598 (**21**), was found to have both DA D_2 and $5HT_2$ antagonist activity,



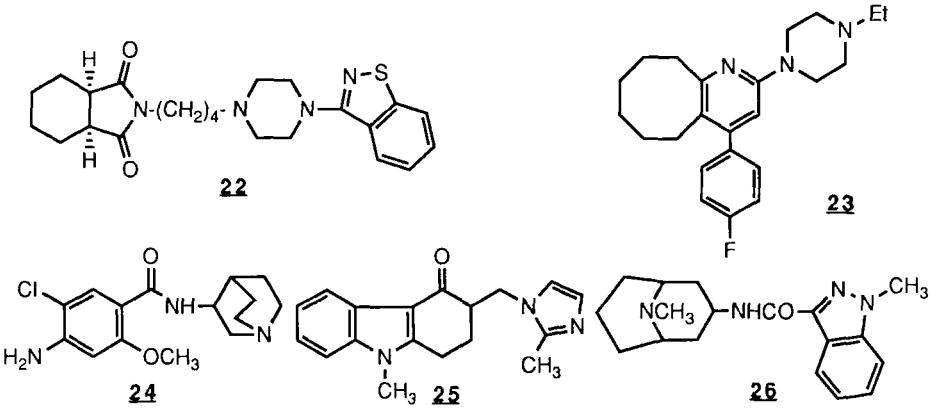
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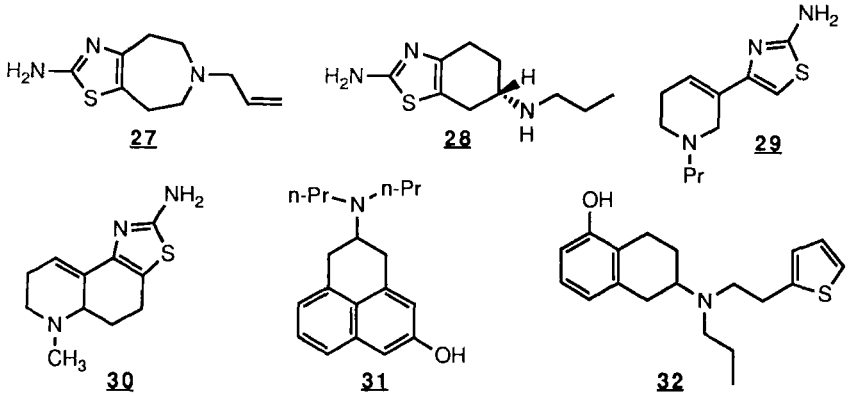
21

greater dopaminergic antagonist potency than risperidone and haloperidol-like effects in preclinical tests (70-72). SM-9018 (**22**), a compound with affinity for both $5HT_2$ and DA D_2 receptors, increased brain DA turnover and inhibited both $5HT_2$ and D_2 receptor-mediated behaviors in rodents (73,74). AD-5423 (**23**) was described as another potent D_2 and $5HT_2$ antagonist with an in vivo biochemical and behavioral profile predictive of antipsychotic activity (75).

The investigation of brain $5HT_3$ receptors represents an additional serotonergic strategy in antipsychotic drug development (76,77). The $5HT_3$ antagonist, zacopride (**24**), was reported to produce little change in positive schizophrenic symptoms in clinical trials (78). Preclinical studies with $5HT_3$ antagonists showed that ondansetron (**25**) reduced limbic DA receptor activation but did not affect DA or 5HT metabolism in brain (79) and that BRL 43694 (**26**) did not produce its antipsychotic-like behavioral effects by altering the firing rate of brain DA neurons (80).



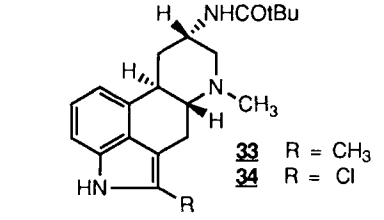
DA Autoreceptor Agonists - The identification of drugs that modulate brain DA neuronal transmission by effects on presynaptic DA autoreceptors remains an active area of research (81). The DA autoreceptor agonist talipexole (B-HT 920, **27**) was evaluated in an open-label clinical trial. Although four patients improved, psychomotor activation was observed in seven patients (82), a profile consistent with preclinical findings of postsynaptic DA agonist-like effects of this agent (83). In rat pituitary, **27** was shown to stimulate a subtype of D_2 receptors linked to voltage-dependent K^+ flux, suggesting alternative signal transduction mechanisms for some brain DA D_2 receptors (84,85). A related aminothiazole, pramipexole (SND 919, **28**), caused a long-lasting decrease in brain DA release (86). Although another aminothiazole, PD 118440 (**29**), lacked selectivity for presynaptic versus postsynaptic DA receptors, the related PD 128483 (**30**) was found to have greater selectivity for DA autoreceptors, to produce antipsychotic-like effects in both rodent and monkey tests and not to cause EPS in monkeys (87,88).



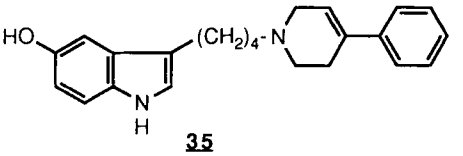
The DA autoreceptor agonist profile of U-68553B (**31**), the (+)-enantiomer of U-66444B, included binding to DA D_2 receptors, inhibition of brain DA synthesis and metabolism, inhibition of brain DA neuronal firing, and reduction of plasma prolactin (89). This compound also appeared to be more selective for presynaptic versus postsynaptic DA receptors than apomorphine and did not induce tolerance after two weeks of repeated dosing (90,91).

Whereas full DA agonists might produce undesirable psychotomimetic effects, certain partial DA agonists can produce antipsychotic-like effects in preclinical tests. Such findings support the proposal that the apparent autoreceptor selectivity of many DA agonists stems from their intrinsic activities at DA D_2 receptors rather than absolute affinities for presynaptic versus postsynaptic DA receptors (92). The (+)-enantiomer of N-0437 (**32**), a partial DA agonist, but not its (-)-enantiomer, a full agonist, was found to produce haloperidol-like behavioral effects, but not catalepsy (93). SDZ 208-911 (**33**) and SDZ 208-912 (**34**) appear to have partial DA receptor agonist properties with antipsychotic-like preclinical profiles (94,95).

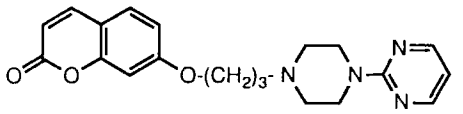
The activities of the indolylbutylamines including roxindole (**35**) were highlighted in a recent review on central DA autoreceptor agonists (96). The SAR of this class of compounds differs from the more classical DA agonists derived from either DA or ergots. The 4-phenyltetrahydropyridine moiety appears to be the most sensitive portion of the molecule to structural modification. An unsubstituted phenyl or thiophene ring as well as the double bond of the tetrahydropyridine appear to be essential for DA agonist activity. Consistent with this, SAR studies on a related series of aminopropoxybenzopyranones, e.g., PD 118717 (**36**), demonstrated that the heterocyclic group and side chain could be varied but the amino portion of these compounds was more sensitive to modification (97). An unsubstituted phenyl or simple heteroaromatic group attached to either a tetrahydropyridine or piperazine ring was required for activity. However, the foregoing does not account for the reported presynaptic selectivity of OPC-4392 (**37**), a compound with 2,3-dimethyl substituents on the phenyl ring of the phenylpiperazine (98,99).



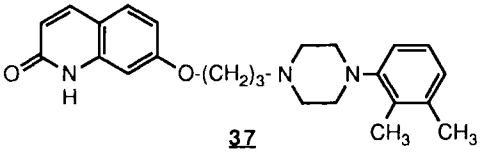
33 R = CH_3
34 R = Cl



35

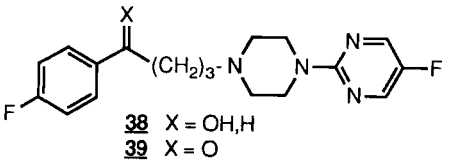


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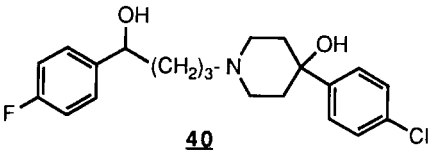


37

Sigma Ligands - Reviews have appeared on the pharmacology and possible functional roles of brain sigma sites (100,101). A study on the enantiomers of the sigma ligand, BMY 14802 (**38**), revealed that the R-(+)-enantiomer had 10-fold greater affinity for sigma binding sites than the S-(-)-enantiomer (102). Other studies compared the effects of (**38**) with those of clozapine (103) and described effects on $5HT_{1A}$ receptors (104) and brain neurotensin (105). Butyrophenone **39** was identified as a major metabolite of **38** in the rat with more of **39** formed from the (-)-enantiomer than from the (+)- enantiomer. Metabolite **39** had modest affinity for DA D_2, alpha$_1$ adrenergic and sigma sites but not $5HT_{1A}$ receptors, results that might explain some of the effects of **38** (106). While haloperidol binds with equal affinity to both sigma sites and DA D_2 receptors, its reduced metabolite (**40**) retained only its sigma affinity. Because **40** was present in the serum of haloperidol-treated patients at levels similar to haloperidol itself (107), these results may have additional implications for sigma involvement in the effects of haloperidol.

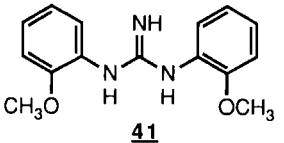


38 X = OH,H
39 X = O

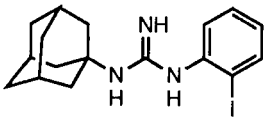


40

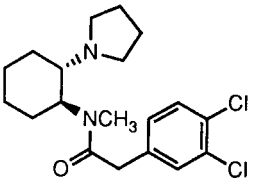
Guanidine 42 was identified as the most potent of a series of sigma ligands related to DTG (41) with an IC_{50} of 3 nM at sigma sites. In general, their affinities for sigma were much higher than for PCP receptors (108). Extensive SAR studies on the kappa agonist, U50,488 (43), led to the identification of the 1S,2R-(-)-diamine 44 with potent sigma affinity and without significant affinity for kappa, DA or PCP receptors (109). A series of benzobicyclic sigma ligands was also described, of which SC-50691 (45) had sub-nanomolar affinity for sigma sites (110).



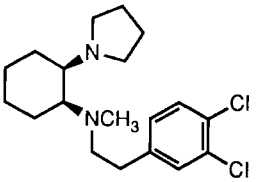
41



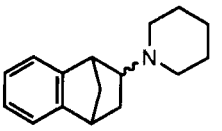
42



43 - (±)



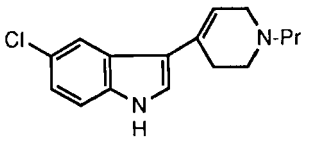
44



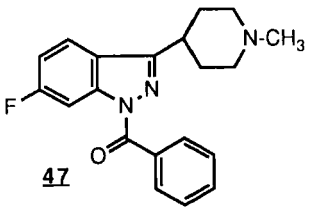
45

Among new findings was evidence for multiple subtypes of sigma binding sites (111,112), microsomal localization of sigma sites (113), the association of sigma binding sites with cytochrome P450 (114), and the possible existence of an endogenous sigma ligand in brain (115). Evidence was also presented that the psychotomimetic effects of benzomorphan opiates stem from actions at PCP receptors rather than from actions at sigma sites (116). Although compounds with sigma affinity have been reported to produce a number of effects that may stem from actions at sigma sites (100,117), the lack of clearly-defined agonists or antagonists for these sites and the use of sigma ligands with multiple receptor activities has hindered the elucidation of their functional role.

Miscellaneous Compounds - Antipsychotic-like effects were described for two compounds of unknown mechanism of action. HR-592 (46) was active in a number of behavioral tests predictive of antipsychotic efficacy (118). HP 818 (47) was reported to be a centrally acting analgesic with antipsychotic-like effects in preclinical tests (119).



46



47

NEW BIOLOGICAL FINDINGS

DA Receptor Clones - The cloning and characterization of multiple DA receptors represented a major new development for antipsychotic drug research. Continued work on the cloned DA D2 receptors characterized two receptor isoforms which appear to be derived from the same gene by alternative splicing and which may differ in signal transduction mechanisms as well as distribution

in brain (120-126). Studies have also begun on the characterization of ligand binding sites of the DA D_2 receptor (126). The DA D_1 receptor has also been cloned and characterization studies reveal DA D_1 receptor mRNA to be highly localized in striatal and limbic forebrain areas (127-129). The cloning of a third type brain DA receptor, termed DA D_3 was also reported (130). The DA D_3 receptor differs in pharmacology and tissue distribution from the previously described DA D_2 and DA D_1 receptors, showed particularly high affinity for DA agonists, and was localized in the limbic forebrain. The availability of expressed DA receptor clones provides powerful tools for exploring the biology of brain DA systems, for understanding drug-receptor interactions, and for identifying selective potential antipsychotics.

Etiology of Schizophrenia - Reviews examined the physical and biochemical alterations in the brains of schizophrenics (131,132). Evidence obtained from postmortem brain tissue suggested an impaired interaction between DA D_1 and DA D_2 receptors in schizophrenia (133). Preclinical studies suggest that neurological side effects of antipsychotics may likewise stem from an imbalance between DA D_1 and DA D_2 receptors (134). A role for hippocampal dysfunction in schizophrenia was proposed (135). Additional evidence was obtained for lateralized hemispheric dysfunction in schizophrenia (136,137). A study suggested that psychotic patients who respond to DA antagonist therapy show pre-drug evidence of elevated brain catecholamine release (138). A correlation was found between negative schizophrenic symptoms and apparent brain norepinephrine release in unmedicated schizophrenic patients who relapsed (139). Several theories have focused attention on the role of brain glutamate in antipsychotic drug action and in the etiology of schizophrenia (140-144). A possible contribution of brain neuropeptides to antipsychotic efficacy was suggested by other studies (40,41).

Animal Models - New preclinical studies on prepulse inhibition provided further support for a model of schizophrenic sensorimotor gating deficits (145). Several findings question the long-standing theory that striatal DA receptor supersensitivity mediates the augmented behavioral responsiveness to DA agonists after chronic dosing with DA antagonists, a proposed animal model for tardive dyskinesia (146-149).

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SECTION II. CARDIOVASCULAR AND PULMONARY AGENTS

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Chapter 7. Angiotensin / Renin Modulators

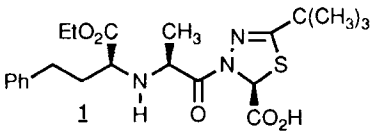
William J. Greenlee and Peter K. S. Siegl
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Introduction - The effectiveness of blocking the renin-angiotensin system (RAS) for the treatment of hypertension has been well established by angiotensin-converting enzyme (ACE) inhibitors. The commercial success of captopril, followed by enalapril and lisinopril, has intensified the search for other ACE inhibitors and for other mechanistically-distinct agents which might block the RAS. Recent introductions of the ACE inhibitors perindopril, quinapril, benzazepril, ramipril and fosinopril increase the options for treatment using this class of agents. Nearly twenty additional ACE inhibitors are in development, and new potential utilities for ACE inhibitors continue to be discovered. The search for specific inhibitors of renin, begun prior to the development of the ACE inhibitors, continues to progress, although the problem of oral bioavailability has not yet been solved. The recent reports of potent, non-peptidic angiotensin II (AII) antagonists and the attendant discovery of AII receptor subtypes has opened a new and promising area for drug discovery in the RAS area. Several reviews outline the general pharmacology of the RAS (1-3).

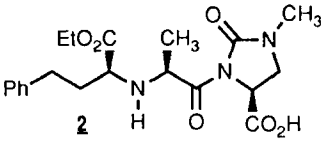
ACE INHIBITORS

Reviews of the medicinal chemistry and pharmacology of ACE inhibitors have appeared (4-6). Currently available ACE inhibitors have shown excellent therapeutic efficacy in hypertension and congestive heart failure. Potential new clinical applications of ACE inhibitors for the treatment of cognitive disorders, cardiac hypertrophy and vascular proliferative disorders have been reviewed (7). Several new ACE inhibitors have longer durations of action than captopril and enalapril. Such agents are likely to be once-a-day therapy in a greater number of patients. Utibapril (FPL 63547; **1**) and imidapril (TA 6366; **2**) are prodrug esters with long duration of action (8,9) For **1**, the greater duration is attributed to a biliary route of elimination (10,11). The long-acting inhibitor ceranapril (SQ29852; **3**) is not a prodrug ester (12).

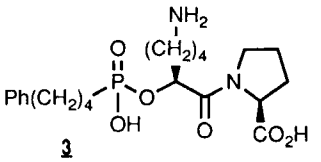
ACE converts angiotensin I (AI) to AII and also participates in the metabolism of several biologically active peptides (13) including bradykinin (BK). Elevation of BK levels with ACE inhibition may contribute to the antihypertensive and cardioprotective actions of these drugs (14). However, several untoward effects of ACE inhibitors, including increased cough reflex and potentiation of inflammation, may be a consequence of elevated BK levels (15,16). AII receptor antagonists and renin inhibitors (see below) offer mechanisms for AII inhibition without a BK component.



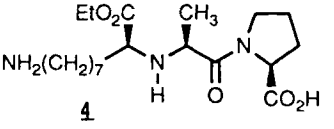
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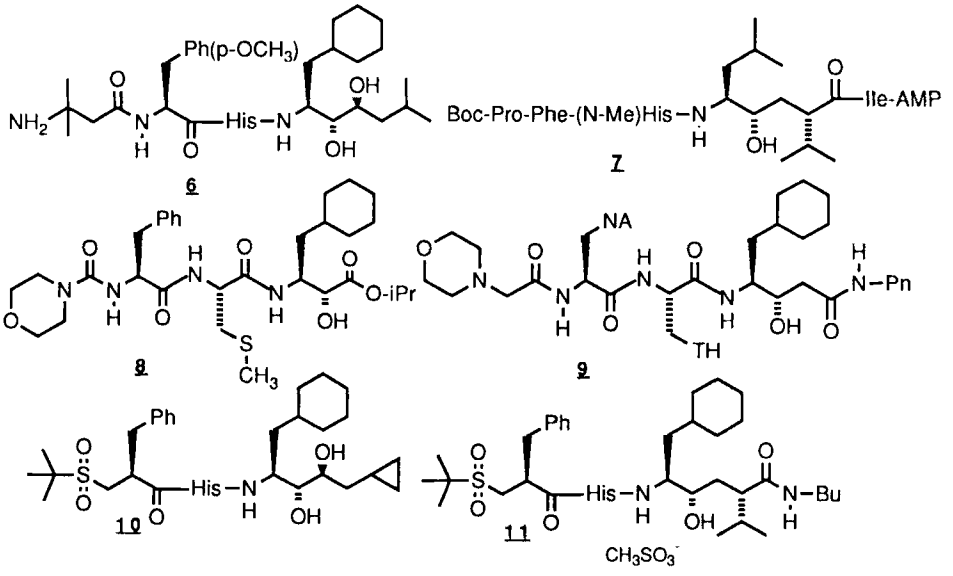
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Inhibitors AB-47 (4) and imidapril (2) are claimed to be more selective as inhibitors of AI to AII conversion (relative to cleavage of bradykinin) than captopril and enalapril (17-19). Since the enzyme inhibited by these compounds is the same, the mechanism for selectivity is unclear, but may be related to tissue pharmacokinetics. A novel, non-competitive octapeptide inhibitor (Pro-Thr-His-Ile-Lys-Gly-Asp), first isolated from tuna muscle (20,21), is found in muscle from several sources and is thought to be derived from glyceraldehyde 3-phosphate dehydrogenase via acid-limited hydrolysis (22). Formation of AII via enzymatic pathways not inhibited by ACE inhibitors adds further complexity to the pharmacology of these drugs (23). A highly specific chymase has been identified and characterized from human heart (24). This ACE inhibitor-insensitive enzyme is reported to be the major pathway for cardiac synthesis (24,25) and plays a (smaller) role in the vasculature (26). It has been proposed that a positive inotropic effect of AII formed via this enzyme when AI levels are elevated by ACE inhibitors contributes to the therapeutic benefit of these drugs in congestive heart failure (27). If true, renin inhibitors and AII receptor antagonists will not share this mechanism for improving cardiac performance. At present there are no known specific inhibitors of this AI specific chymase and no obvious therapeutic utility for inhibitors.

RENIN INHIBITORS

Clinical Pharmacology - Several reviews (28-31) summarize recent developments in the design of renin inhibitors (RI). While studies of renin antibodies and of active immunization against renin have established that inhibition of renin within the plasma compartment is sufficient to lower blood pressure (BP) for an extended period (32,33), recent clinical studies of renin inhibitors have reinforced initial findings in animal studies which implicate tissue renin as an important factor in mediating the response of blood pressure to RI's. Clinical data are available for six RI's. In hypertensive patients, enalkiren (6) completely inhibited plasma renin activity (PRA) after intravenous administration, but suppression of BP required 400-1,000-fold higher doses (34,35). It was suggested (36) that release of enalkiren from tissue depots may explain why prolonged renin inhibition results despite a short plasma half-life ($t_{1/2}$ = 1.9 hours). There was no loss of antihypertensive effect during one week of dosing, but oral bioavailability was only 0.5% after a 40 mg dose (37). Enalkiren applied topically lowered intraocular pressure in humans (38). Intravenous administration of enalkiren produced hemodynamic benefits in patients with chronic heart failure (39).

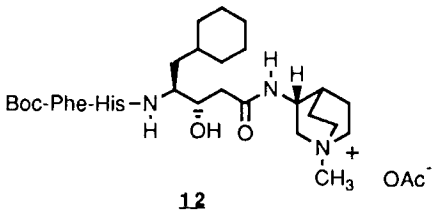


AMP = -CH_2(pyridin-2-yl) NA = 1-naphthyl TH = thiazol-4-yl Pn = n-pentyl

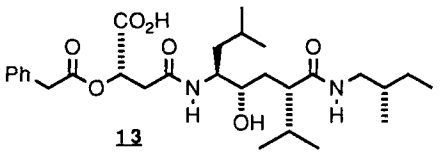
Intravenous infusions of ditekiren (7, U-71,038) lowered PRA and produced a dose-related fall in BP which was greater in salt-depleted patients (40). CP-80,794 (8) inhibited diuretic-

stimulated renin secretion for up to 6 hours in normotensive males after single oral doses (41). After oral administration of ES-8891 (**9**) to normotensive humans, plasma levels of drug and inhibition of PRA persisted for over 2 hours, without significant changes in BP (42). PRA and BP were lowered after either i.v. or oral administration of Ro-42,5892 (**10**) to hypertensives, and BP remained low for several hours after the oral dose. The effects on BP were maintained over a longer period than suppression of AII levels, suggesting an action independent of suppression of circulating AII. RO-42,5892 is the first renin inhibitor to show prolonged suppression of BP in patients without sodium depletion, and the first shown to have significant activity when administered orally (43). The inhibitor CGP-38,560A (**11**) lowered BP and AII levels, but the time course of both was dissociated from PRA levels. The reduction in BP did not equal that produced by captopril, in spite of dramatic drops in plasma AI and AII levels and circulating levels of inhibitor 1000-fold higher than its K_i value. Oral bioavailability was less than 1% (44,45).

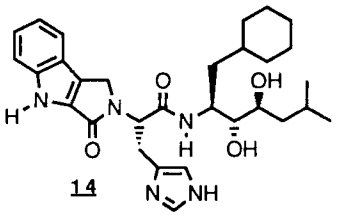
New Inhibitor Designs - Potent renin inhibitors which incorporate a modified carboxy-terminus have been reported, including 1,3-diols (46,47), homologated aminoalcohols (48) and hydroxyphosphonates (49). Inhibitors such as **12**, containing a carboxy-terminal quaternary ammonium salt have longer duration of action than the corresponding free bases, possibly due to slower biliary extraction (50). Others contain amino-substituted heterocycles replacing histidine at P_2 (51). In the potent (IC_{50} = 5 nM) and low molecular-weight (MW=535) inhibitor **13**, an acylated malic acid replaces the traditional amino-terminal acylated dipeptide element (52).



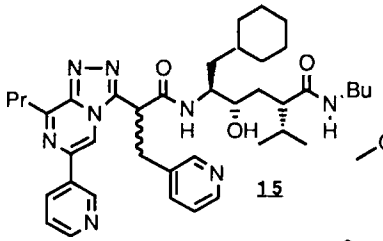
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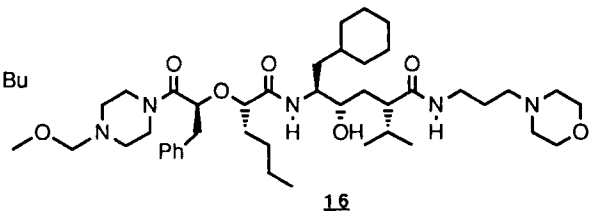
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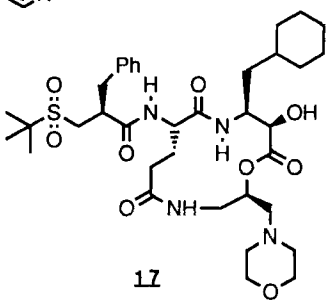
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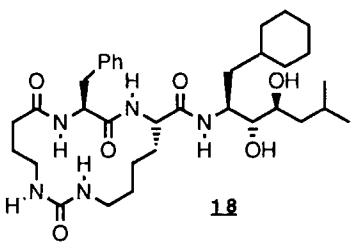
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18

The introduction of heterocyclic elements serves to eliminate amino-terminal amide bonds in lactam **14** and the bicyclic heterocycle **15** (53,54). Boronic acid inhibitors designed as potential prodrugs of the corresponding alcohols are themselves inhibitors of renin (55). Inhibitor **16** which incorporates a novel amino-terminal ether linkage lowers BP for 5 hours in sodium-depleted cynomolgus monkeys after an oral dose of 3 mg/kg (56). Potent macrocyclic renin inhibitor **17** links P_2 and P_1' elements in a 13-membered ring, while inhibitor **18** (which is stable to chymotrypsin) links P_2 and P_4 elements in a 16-membered ring (57,58). Potent cyclic peptide inhibitors of renin such as Me_3CCH_2-cyclo[Glu-D-Phe-Lys]-D-Trp-$NH(CH_2)_2$-iPr bear little resemblance to human renin substrate (59). Replacement of the basic imidazole ring of histidine at P_2 with a neutral thiazoline ring improved intestinal absorption (in rats) in a series of potent inhibitors (60). Interaction of renin inhibitors with the intestinal uptake system for oligopeptides and β-lactam antibiotics has been reported (61).

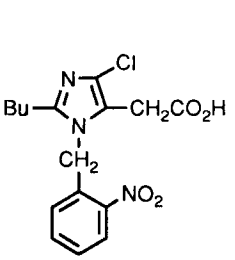
ANGIOTENSIN II ANTAGONISTS

In Vitro Pharmacology - Recent reviews (62-64) of direct receptor antagonists of AII (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) describe the development of the peptide antagonists saralasin (Sar-Arg-Val-Tyr-Val-Ile-His-Pro-Ala), [Sar^1, Ile^8]AII, and [Sar^1, $Tyr(OMe)^4$]AII (sarmesin). Although saralasin was shown to lower BP in humans with high renin levels (65), partial agonist activity and lack of oral bioavailability limit the the use of peptide antagonists as antihypertensive agents. However, recent work on peptide antagonists has helped define the minimum peptide sequence required for binding and agonist activity and aided in conformational analysis of AII. Analogs of Sar^1-AII have been reported in which Phe^8 has been replaced by aromatic amino acids with increased bulk or conformational restraint. A highly hydrophobic analog of AII, [Sar^1, (2',3',4',5',6'-Br_5)Phe^8]AII, is a potent antagonist of AII both in vitro and in vivo. Its prolonged duration of action may be due to slow dissociation from the receptor (66,67). Analogs in which Phe^8 is replaced with diphenylalanine (Dip), (biphenyl-4-yl)alanine (Bip) or 2-aminoindane-2-carboxylic acid (2-Ind) have also been reported. In a rat uterus contraction assay, both [Sar^1, Dip^8]AII and [Sar^1, D-Dip^8]AII are potent agonists, while [Sar^1, Bip^8]AII, [Sar^1, D-Bip^8]AII and [Sar^1, 2-Ind^8]AII are potent antagonists (68). Both [Dmp^8]AII and [Sar^1, Dmp^8]AII (DMP = 2,6-dimethylphenylalanine) are potent AII antagonists in rabbit aorta (69). AII analogs truncated at either the amino- or carboxy-terminus have been investigated. Both [Sar^1]A(1-7)-NH_2 (pA_2 = 7.6) and [Sar^1]A(1-6)-$N(CH_3)_2$ (pA_2 = 7.0) were competitive, reversible receptor antagonists in the rabbit aorta, and antagonists of AII pressor response in rats at doses only 6 to 10-fold higher than for saralasin (70,71). A series of amino-terminally modified hexa and pentapeptides, including δ-Ape-Tyr-Ile-His-Pro-Ile (Ape = 5-aminopentanoyl), are antagonists of AII in rabbit aorta (72). None of the truncated AII analogs show agonist activity. Cyclic AII analogs have been prepared by connecting the side-chains of homocysteine (Hcy) residues at positions 3 and 5 via a disulfide bridge. The analog [$Hcy^{3,5}$]AII has excellent binding affinity to rat uterine membranes and high contractile activity on rabbit aortic rings (73). A cyclization of this type was used to prepare a highly potent antagonist [Sar^1, $Hcy^{3,5}$, Ile^8]AII, which model building suggests contains a 3-residue turn in its bioactive conformation (73,74).

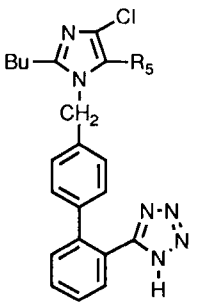
The first non-peptidic AII antagonists described were benzyl-substituted imidazoles (75,76) of which the selective and competitive antagonist S8308 (**19**) is an example (62). Higher potency has been achieved with DuP753 (MK-954; **20**), an orally-active antagonist in clinical trials (see below). The biphenyl element appears to be important for oral activity since analogs with amide and other linkers between the phenyl rings are not orally active (77,78). EXP3174 (**21**), a major metabolite of DuP753 formed in rats, is a potent AII antagonist in rabbit aorta (79). Antagonists in which other 5-membered ring heterocycles replace the substituted imidazole of DuP753 (80-82) include the pyrazole **22** and the pyrrole **23**. Antagonists incorporating triazoles (80,83) benzimidazoles (84-88), purines (89,90), imidazo[4,5-b]pyridines (89,90) and other fused imidazoles (91,92) have been disclosed, as have pyrimidinone (93,94), quinazolinone (93,94) and triazolinone (95) antagonists. Hybrid AII antagonists such as **24** which incorporate beta-blocking activity have been disclosed (96). Such an antagonist might prevent the compensatory rise in renin levels expected to result from blockade of AII receptors, a response which can limit the efficacy of agents which block the RAS (97). Imidazoleacrylate antagonists exemplified by **25** have also been disclosed (98).

The availability of non-peptidic antagonists has permitted the study of AII receptor subtypes in numerous tissues. DuP753 and EXP3174 have high selectivity for a receptor subtype

found in several tissues, including aorta, liver and adrenal (Table 1). Other antagonists with high selectivity for a second receptor subtype found in adrenal, uterus and brain have been reported (99-103). These include PD-121981 (**26**, WL-19), PD-123177 (**27**, EXP655), PD-123319 (**28**) and PD-124125 (**29**). Selectivity for this receptor has been reported for the peptides CGP-42112A **30** and [Sar^1,Phe(4-NH_2)^6]AII (104,105). It has been proposed that these two AII receptor subtypes be designated AT_1 and AT_2 (Table 1), a nomenclature which would replace others utilized previously (106).

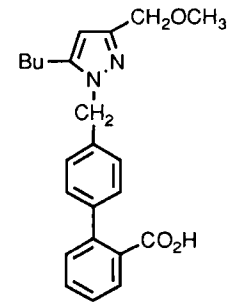


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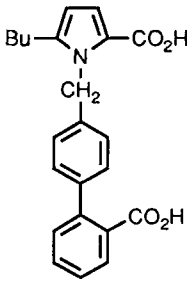


20 R_5 = -CH_2OH

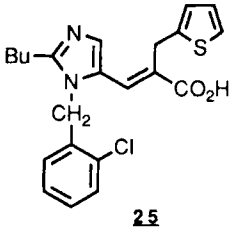
21 R_5 = -CO_2H



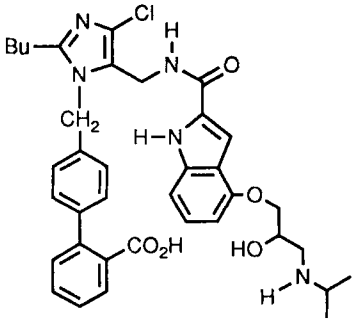
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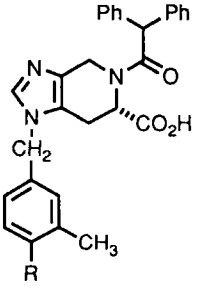
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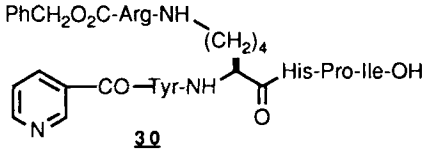


26 R = - OCH_3

27 R = -NH_2

28 R = -$N(CH_3)_2$

29 R = -H



30

Angiotensin II receptors occur in many tissues including vascular smooth muscle, kidney, adrenal, liver, and brain (111). Receptors for AII have not been well-characterized biochemically, and no proteins established to be AII receptors have yet been cloned and sequenced. The mas oncogene was reported to encode an angiotensin receptor (112), but binding of AII to mas-transfected cells has not been demonstrated (113).

Table 1 AII Receptor Subtypes

RECEPTOR	ANTAGONISTS	REFERENCES
AT_1 (AII-1, AII-B, AII_α)	DuP753, EXP3174	102, 105, 106
AT_2 (AII-2, AII-A, AII_β)	PD-123177,	102, 107, 108
	PD-123319,	106
	PD-121981,	109, 110
	CGP-42112A,	105, 106
	[Sar^1, $Phe(4\text{-}NH_2)^6$]AII	105

Tools for receptor characterization include [^{3}H]-DuP753, a specific radioligand for the AT_1 receptor subtype (114). A biotinylated and radioiodinated analog of AII (Biotin-$CONH(CH_2)_2$-$SS(CH_2)_2CO$-[Ala^1,$Phe(4N_3)^8$]AII) for photoaffinity labelling and purification of AII receptors may allow isolation of AII receptors (115,116). Other receptors for AII have been characterized using non-peptidic antagonists. Nuclear AII binding sites in rat liver cells which bind DuP753 are distinct in physiochemical properties to the membrane-bound receptors, and may mediate intracellular actions of AII (117). A cytoplasmic AII binding protein in rat liver cells (118) binds neither DuP753 nor PD-124125 (**27**). Evidence for two distinct receptors for AII in human astrocytes, one of which is blocked by DuP753, has been reported (119). Evidence for the existence of AT_2 receptors in neuronal cells and their upregulation during differentiation has been reported (120). Selective AII antagonists (DuP753 and CGP-42112A) have revealed differing developmental patterns for AII receptors in rat brain. While AT_1 receptors were predominant in adult (8-week-old) rats, AT_2 receptors were expressed remarkably in young (2-week-old) rats (121). The AT_1 receptor is reported to be coupled to the phospho-inositide turnover transduction system (122), but no second messenger system has yet been identified for the AT_2 receptor.

Introduction of conformational restriction or cyclizations into AII peptide analogs has provided the basis for predictions about the bioactive conformation of AII. NMR studies of [Sar^1]AII in dimethylsulfoxide, which suggest clustering of the three "aromatic" sidechains of Tyr^4, His^6 and Phe^8, were used to propose a solution conformation (123). A carboxy-terminus receptor model was derived from a study of antagonists derived by D-aminoacid substitutions for Phe^8 of [Sar^1]-AII (124). Models which overlay non-peptidic antagonists onto putative conformations of AII (77,125) place the imidazole ring of DuP753 over His^6 and orient the biphenyltetrazole toward either Asp^1 or toward Phe^8. An extensive list of citations of studies of AII conformations has been published (77). An X-ray crystal structure of the amino-terminal tetrapeptide of AII (Asp-Arg-Val-Tyr) has been reported (126).

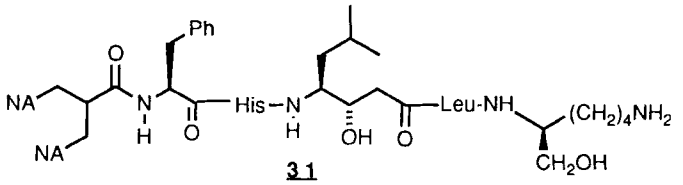
In Vivo Pharmacology - In normotensive rats, orally-administered DuP753 blocked the pressor response to AII, and blocked AII-induced drinking response and aldosterone release (127). In sodium-depleted rats, acute administration of DuP753, enalaprilat or a renin inhibitor produced equivalent effects on BP (128). In spontaneously hypertensive (SH) rats, DuP753 gave an equivalent reduction in BP to enalaprilat (113), but produced a greater BP response than did captopril in another acute study (129). Oral administration of DuP753 to normotensive humans produced a dose-dependent inhibition of the pressor effect of AII and a dose-dependent rise in PRA, with no evidence of an agonist effect. Repeated administration of DuP753 over several days enhanced the compensatory rise in renin secretion, and plasma renin was still elevated 24 hours after the last dose. (130,131,97). Anxiolytic-like activity has been reported for DuP753 after oral administration to rats (132).

A function for the AT_2 receptor has yet to be identified. The very specific distribution of ^{125}I-AII or ^{125}I-[Sar^1, Ile^8]-AII binding at AT_1 vs AT_2 sites between and within tissues is suggestive of different functional responses for each binding site. For example, AII binding sites are predominately AT_1 in rat adrenal cortex , while those in the rat adrenal medulla are predominately AT_2 (102, 108). The selective AT_1 antagonist DuP753 inhibits both AII-stimulated aldosterone release from adrenal cortex (108,127) and AII-stimulated catecholamine secretion from adrenal (102), while selective AT_2 antagonists (eg. **26**-**29**) have no effect on either response. In other studies, no effects of AT_2 compounds were found on contractile effects of AII in rabbit aorta and

uterus, AII pressor and tachycardic response, AII-induced drinking response, or blood pressure in renal artery-ligated (hypertensive) rats (133-135). Elucidation of a physiological role, if any, for AT_2 receptors is of high interest, and further developments are awaited.

FUTURE DIRECTIONS

Recent reports of an enzyme responsible for the activation of prorenin by a specific cleavage of a 43-amino acid prosegment ("renin processing enzyme" or "prorenin convertase") present yet another potential target for specific blockade of the RAS, and medicinal chemistry has begun in this area (136,137). Future targets within the RAS also include inhibition of the DNA-binding activity of specific trans-acting renin-gene activators or enhancers (138). A renin inhibitor **31** (ES-1005) which also suppresses expression of the kidney renin gene has been reported (139,140). Transgenic mice which carry both rat renin and rat angiotensinogen genes have elevated systolic blood pressure which can be reduced with captopril (141). Transgenic mice carrying the human renin gene (142) may be useful in evaluation of inhibitors of the RAS.



31

NA = 1-naphthyl

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Chapter 8. Potassium Channel Activators

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Introduction - The first review in this series (1) on potassium channel activators (KCAs), or openers, detailed the enormous growth of interest in the area since the elucidation of the mechanism of action of cromakalim (1), namely enhancing the movement of potassium ions through smooth muscle cell membranes. This chapter summarizes the continuing high level of interest in the physiology and pharmacology of K^+ channels, the structure-activity relationships of compounds that modulate such channels, and the diverse indications described for these agents. Useful reviews on K^+ channels and KCAs have appeared (2-7).

Physiology of Potassium Channels - The continuing characterization of K^+ channels has been due to advances in molecular biology and electrophysiology techniques. Studies examining the degree of heterogeneity among K^+ channels have resulted from the continued identification of high affinity probes, notably venom toxins, which act as selective blockers of K^+ channels (8).

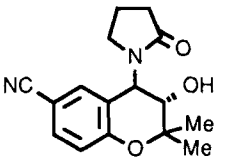
Initial advances in our knowledge of the structure of K^+ channel proteins is a consequence of the characterization of "Shaker" gene mutants in Drosophila which encode a voltage-dependent K^+ channel (transient outward, A-type current). Subsequently, a K^+ channel from rat brain has been cloned and appeared to be homologous with "Shaker" K^+ channels. Other genes for K^+ channels in the CNS have been cloned, expressed, and studied in Xenopus oocytes, and appear to represent voltage-dependent K^+ channels which carry current of the delayed rectifier type (9). The pharmacological profile of these K^+ channels has been studied using blockers of the channel subtypes, such as tetraethylammonium and 4-aminopyridine, in addition to a variety of toxins, including mast cell degranulating peptide (MCDP), dendrotoxin, β-bungarotoxin, and noxiustoxin (8). High affinity probes such as toxins are currently used to identify structural features of the K^+ channel, for instance to determine if the channel protein is constituted from a number of subunits. Evidence for the purification and characterization of low conductance (apamin-sensitive) and high conductance [charybdotoxin (CTX) and noxiustoxin-sensitive] calcium (Ca^{++})-activated K^+ channels is limited. High conductance Ca^{++}-activated K^+ channels from rabbit aortic smooth muscle have been isolated and incorporated into planar lipid bilayers (10). ATP-sensitive K^+ (K_{ATP}) channels from the insulin secreting cell line H1T T15 have been expressed in Xenopus oocytes (11).

Following identification of K^+ channels that are inhibited by intracellular ATP (i[ATP]) in tissues such as pancreas, heart and CNS (12), recent electrophysiological studies, using inside-out membrane patches, have demonstrated the existence of these channels in vascular and airways smooth muscle. Thus, K_{ATP} channels have been identified in rabbit mesenteric artery (13) - single channel conductance of 135 picoSiemens (pS), porcine coronary artery - 11 pS (14), rat portal vein - 10 pS (15,16), and rabbit and bovine tracheal smooth muscle - 30 to 39 pS (17). In addition to their sensitivity to i[ATP], these channels are also inhibited by glibenclamide. These reports of the identification of K_{ATP} channels in vascular and airways tissue are of significance because KCAs demonstrate efficacy as smooth muscle relaxants in these tissues.

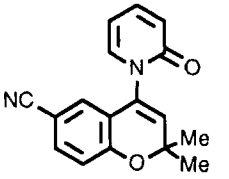
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Since the pharmacological actions of KCAs are inhibited by sulfonylureas, it is of interest that K_{ATP} channels have been identified in the rat substantia nigra, an area containing the highest density of sulfonylurea receptors in the brain (18). Interestingly, ATP-insensitive glibenclamide-sensitive K^+ channels have been identified in rabbit vascular smooth muscle (19) and human skeletal muscle (20).

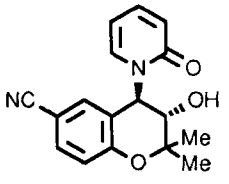
Structure-Activity Relationships in KCAs - Preliminary accounts of this topic deal principally with the benzopyran based KCAs (21-23). [In these compounds, racemates are shown with a normal bond at C(4), whereas single enantiomers are depicted with a tapered bond]. A major part of such studies has been concerned with the modification or replacement of the lactam group at C(4) of cromakalim, and the resultant effect on activity, usually in rat models of hypertension. Unsaturated lactam groups, particularly the 2-pyridone group which confers greater potency than the pyrrolidinone group, have been incorporated at this position (24,25). Examples of such KCAs are EMD 52692 (2) and EMD 56431 (3), which are being developed for angina pectoris and hypertension (26). Incorporation of heteroatoms in the unsaturated lactams, in general lowered activity (24).



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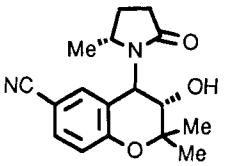


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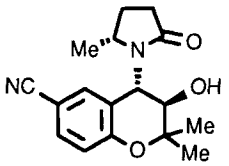


3

An interesting variation is the insertion of the methyl group at C(5′) of the pyrrolidinone ring. The 3S,4R,5′R/3R,4S,5′S racemate (4) is twice as potent as cromakalim in lowering blood pressure (27,28). As the activity of the latter resides predominantly in the 3S,4R enantiomer, it is presumed that the activity of (4) resides in the 3S,4R,5′R enantiomer, and thus the 5′R methyl group enhances potency. The (-)-3R,4S,5′R enantiomer S 0121 (5) which contains the C(3/4) stereochemistry not associated with hypotensive activity, reduced spontaneous tone of guinea pig isolated ureter smooth muscle, and thus may ease the passage of kidney stones.



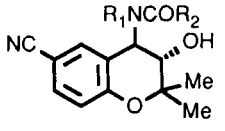
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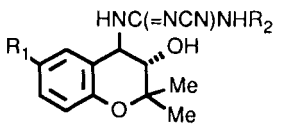
5

The lactam ring can be replaced by acyclic amide groups. Optimal activity was found for the acetamide (6, R_1=H, R_2=Me), whereas the formamide (6, R_1=R_2=H) and analogues with larger R_1 and R_2 groups were less potent. High potency was also found (29) for benzamides (6, R_1=H, R_2=Ph), ureas (6, R_1=H, R_2=NHMe) and cyanoguanidines (30) such as compound (7, R_1=Et, R_2=Me), although the phenyl cyanoguanidine (7, R_1=CN, R_2=Ph) possessed little vasodilator activity, while displaying anti-ischaemic properties (31). Other C(4) replacements include acylated hydrazines (32), for example (6, R_1=NHCOMe, R_2=Me), and the cyanoiminothiazolidine in FR 119748 (8) which was twice as potent as cromakalim (33).

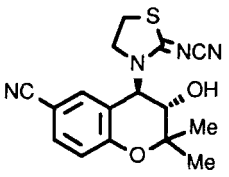
Oxygen linked moieties at C(4) are exemplified by SDZ PCO 400 (9) and EMD 57283 (10). The former was described as equipotent with cromakalim, with a longer $T_{1/2}$ in a monkey model (34), and the latter to be more potent and longer acting than cromakalim in a rat model (35). The C(4) substitutuent in 10 and the 2-oxo-1-methyl-4-pyridyloxy group confer optimal activity in the series. It is notable that these compounds contain carbonyl groups that differ in spatial orientation from that of the cromakalim structure. In further developments the carbonyl group has been removed and activity



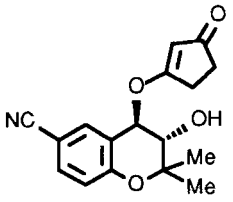
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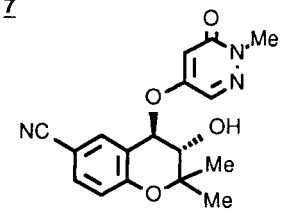
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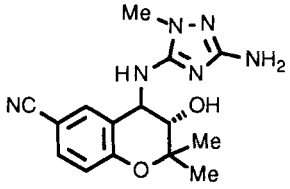


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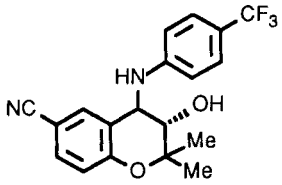


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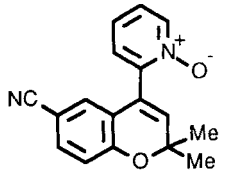
retained. Thus, C(4)-amino linked heterocycles such as the aminotriazole (11) were found to be active antihypertensive agents (36). In contrast, certain C(4) anilino chromans and chromenes, such as SR 46276 (12), have been described as useful antidepressants, with little cardiovascular activity (37). Heteroaryl substituents have been attached directly at C(4), as in the pyridine N-oxide Ro 31-6930 (13) which was ten times more potent than cromakalim (38).



11

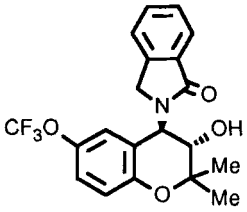


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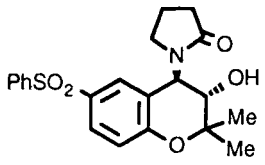


13

The range of electron withdrawing groups at C(6) in benzopyran KCAs that confer high potency has been extended with trifluoromethyl (39) and pentafluoroethyl (40) substituents that confer high potency, and the trifluoromethoxy group of WAY 120491 (14), which is less potent than cromakalim (41). Surprisingly, chromanols containing alkyl groups at C(6) have been found with significant levels of activity which appear to depend on the dimensions of the substituent (39). Phenyl group attachment at C(6) provided modest activity (39), but a phenylsulfonyl group as in HOE 234 (15), raised potency to a higher level than that of cromakalim (42).



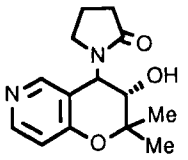
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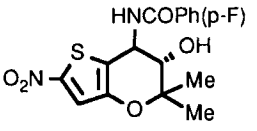
15

Heteroaryl replacements for the phenyl ring of benzopyran KCAs have been introduced, for example the pyrano[3,2-c]pyridine (16). The position of the N atom was found to markedly influence activity (39), as the 5-, 7- and 8-aza analogues were much less potent than (16). Substituted thiophene replacements have been disclosed, in particular the 2-nitrothiophene (17), where the nitro group plays a crucial role, as the compound lacking this substituent is about a hundredfold less potent (43). The 6-cyanophenyl group of cromakalim has been replaced by the benzoxadiazole ring system of NIP 121 (18) which is about an order of magnitude more potent than cromakalim (44).

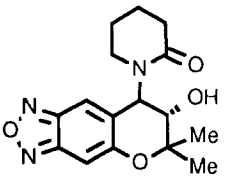
In the 6-methyl analogue of cromakalim, replacement of the pyran oxygen atom by a



16

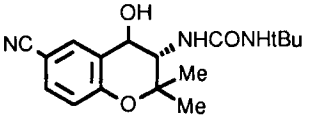


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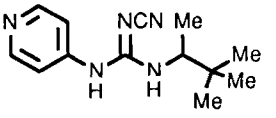


18

sulfur atom maintained activity, but oxidation to the sulfoxide or sulfone gave less potent compounds (45). An interesting development in benzopyran-based KCAs was the discovery that the combination of a substituted urea at C(3) and a *trans* C(4) hydroxyl provides compounds such as (**19**) with similar potency to cromakalim (46).

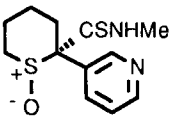


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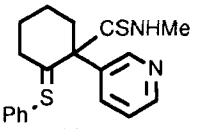


20

Substituted phenyl groups can replace the pyridine nucleus of pinacidil (**20**) but no activity data were presented (47). The studies leading to the discovery of RP 49356 (**21**) have been published, and indicate that the oxidative state of the sulfur atom, and the 1,2-stereochemistry are critical features for activity, which was associated with the (-)-1R,2R enantiomer, RP 52891. Replacement of the pyridine ring of **21** by a 3-quinoline ring enhanced potency fourfold (48). Further studies (49-51) showed that pyridine group replacement by substituted phenyl groups can retain activity, while replacement of the sulfoxide group can provide highly potent compounds *in vitro*, such as (**22**).



21

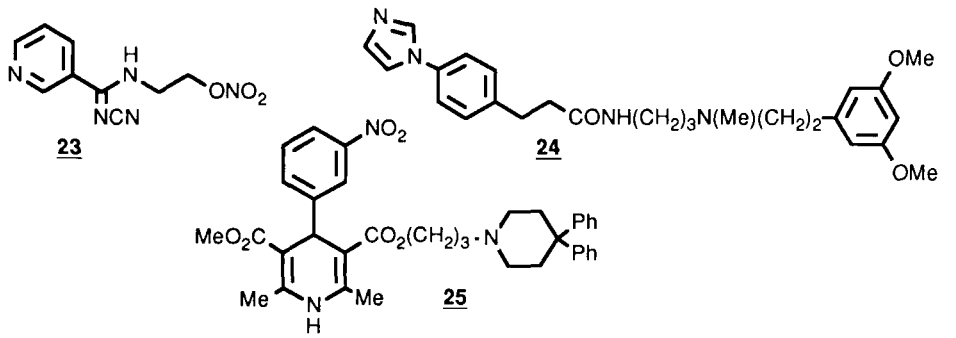


22

X-ray crystallography and NMR studies on cromakalim indicated two low energy conformations of the molecule (52,53). These conformations, with the pyrrolidinone ring disposed in an orthogonal manner with respect to the benzopyran nucleus, can interconvert by rotation about the C(4)-N bond. The ratio of the rotamer populations was altered in the piperidinone analogue, while a 5′-methyl group on the pyrrolidinone ring raised the barrier to rotation about the C(4)-N bond. Amide-bond isomerism in tertiary amide **6** (R_1=R_2=Me) may be responsible (28) for the lower potency compared with secondary amide **6** (R_1=H, R_2=Me).

PHARMACOLOGY

Vascular Smooth Muscle - KCAs inhibit spontaneous tone in portal vein, or contractions evoked by a range of spasmogens in arterial preparations. In spite of sharing some properties with nitro-containing vasodilators, the ability of KCAs to inhibit contractions to low concentrations (≤ 20 mM) as opposed to high concentrations (~ 80 mM) of KCl can be used as a means of discriminating KCAs from other smooth muscle relaxants such as dihydropyridine (DHP) Ca^{++} antagonists. Using these criteria, compounds **2**(54), **13**(55), **9**(56), **14**(57), **18**(58), and **15** (59) have been classified as KCAs. Like the earlier KCAs, K^+ channel opening activity of these compounds has been confirmed by their ability to produce hyperpolarization and enhanced K^+ conductance. These actions of KCAs are not considered to be endothelium dependent, although endothelial effects may play a role in the relaxant properties of **15** (59). KRN 2391 (**23**) inhibits contractions to both high and low concentrations of KCl, as does E 4080 (**24**), suggesting a mixed profile of action for both compounds (60,61), and a possible similarity to niguldipine (**25**), the KCA/Ca^{++} antagonist (62).



The rationale for an association between the vasorelaxant and hyperpolarizing action of KCAs, and consequent reduction in the opening of voltage-sensitive Ca^{++} channels has been reviewed (1). Fluoroscopy studies (63,64) have revealed that KCA-induced relaxation may be a consequence of reduced intracellular Ca^{++} ion concentration (i[Ca^{++}]). However, evidence that the relaxant properties of pinacidil at high concentrations (~ 100 μM) may not be totally dependent upon reduced i[Ca^{++}] (65), or that cromakalim (1 μM) may hyperpolarize rat aorta only in a depolarized state (66), highlights the deficit in knowledge regarding an interaction between KCAs, voltage-sensitive Ca^{++} channels and signal transduction. Although KCAs do not interact directly with DHP binding sites, the hyperpolarization induced by cromakalim may produce a change in the conformational state of voltage-sensitive Ca^{++} channels in rat portal vein and inhibit ^{3}H-desmethoxyverapamil binding (67). Binding of this ligand appears to be highly voltage-sensitive since depolarization produced similar effects to hyperpolarization.

The haemodynamic profiles of recent KCAs have been described following acute administration to hypertensive and normotensive animals (34,38,41,44,59,60,68-72). KCAs **9**,**13**, and **18** retain their hypotensive properties upon repeat dosing (34,38,44). The development of genetic hypertension in young spontaneously hypertensive rats was not attenuated (73) by cromakalim or SR 44866 (**2**). Although the incidence of tachycardia varies among KCAs, the inhibition of this response by β-blockers indicates that it is a reflex mechanism (44,69,72). This tachycardia does not appear to be a consequence of direct stimulation of cardiac tissues as judged by the marked vascular selectivity of these agents (70).

Differences appear to exist in the regional haemodynamic profile of new KCAs which may reflect some selectivity for certain vascular beds. Increased skeletal muscle (femoral), cerebrovascular and gastrointestinal blood flow has been found for a number of KCAs (34,38,68,70-72,74). Marked increases in cerebrovascular blood flow were reported (70) for **2**. Cromakalim displayed more selectivity for decreasing renal vascular resistance than **13** in anaesthetised normotensive rats (68). In the non-ischaemic state, enhanced coronary blood flow was observed (71,72,74,75,) for a number of KCAs; **2** and **23** were more potent than nicorandil or cromakalim (71,74). This suggests that KCAs may have potential as anti-ischaemic agents, particularly if they are able to enhance blood flow to the ischaemic subendocardial layers of the myocardium. Although pinacidil reduced blood flow to the ischaemic subendocardium in conscious dogs (76), enhanced blood flow to both subepicardium and subendocardium was reported for **2** (77). Furthermore, cromakalim, pinacidil, RP 52891, **3** and **23**, improved reperfusion function in ischaemic rat hearts or canine myocardium (78-81). The actions of **23** were only partly glibenclamide sensitive, thus confirming that it may possess multiple pharmacological activities (81).

KCAs have been observed to influence a number of K^+ currents in vascular tissue. Evidence for an involvement of high conductance Ca^{++}-activated K^+ channels has come from studies using the K^+ channel blocker CTX (1), and also the ability of cromakalim to enhance conductance through such K^+ channels incorporated into planar lipid bilayers

(10). Other reports have implicated a delayed rectifier type K^+ current (19,82). However the consistent ability of glibenclamide to inhibit the hypotension (44,72) or *in vitro* actions of KCAs (57-59, 61), has suggested an important action of these compounds upon K_{ATP} channels. Recent electrophysiological evidence demonstrated that cromakalim, nicorandil and pinacidil open such channels, by influencing channel "open state probability" (13-16). This is supported by evidence (83) that cromakalim-induced K^+ channel opening in vascular smooth muscle cells, demonstrated by an enhanced $^{86}Rb^+$ efflux, was associated with modest decreases in i[ATP]. The necessity to use high concentrations of glibenclamide in vascular tissue, highlights the diverse characteristics of these channels in different tissues (12). The association between KCAs and K_{ATP} channels requires further elucidation, particularly since cromakalim has been reported to open glibenclamide-sensitive, ATP-insensitive K^+ channels in vascular tissue (19).

Cardiac Tissue - KCAs shorten action potential duration (APD) and produce a negative inotropic effect in cardiac tissue (1). These events were observed at concentrations higher than those required to produce vasorelaxation, indicating a selectivity for vascular smooth muscle. The ability to shorten APD and to decrease automaticity to arrhythmogenic stimuli has suggested that KCAs may have potential as antiarrhythmic agents (1).

Current evidence is equivocal regarding the nature of KCA action in cardiac tissue. Nicorandil produced antiarrhythmic actions by inhibiting automaticity in canine isolated Purkinje fibres (84). In anaesthetized rabbits, BRL 38227, the 3S,4R-enantiomer of cromakalim, reduced incidence and duration of fibrillation during myocardial ischaemia, and also during reperfusion (85). A beneficial profile of KCAs is supported by evidence of improved reperfusion function (78,79) and minimal influence upon levels of high energy phosphates (86,87). However, KCAs have demonstrated arrhythmogenic activity during ischaemia or reperfusion in rat isolated hearts (88,89), while the K^+ channel blocking sulfonylureas proved to be antiarrhythmic (89). Although atrial arrhythmias were induced by cromakalim and pinacidil in conscious dogs the effect was observed at doses producing excessive ($\geq$ 40%) hypotension (90).

The cardiac effects of KCAs may be explained by an action on K_{ATP} channels (91). Although KCAs are considered to act by a common mechanism, diazoxide has been shown to act as a K^+ channel blocker by inhibiting ATP-sensitive K^+ current and prolonging APD in the rat ventricular epicardium (92). It has been reported that KCAs such as cromakalim, or RP 49356, open K_{ATP} channels by raising the i[ATP] required for half-maximal inhibition of channel activity, suggesting competition between KCAs and ATP for a common binding site (93). Thus, in conditions such as ischaemia, KCAs may influence cardiac electrical and mechanical activity at lower concentrations than used to date. More studies are required to ascertain if there is a cardioprotective role for KCAs.

Airways - In guinea pig isolated trachea, or human bronchial smooth muscle, KCAs relaxed spontaneous tone or tone induced by a range of spasmogens including histamine, carbachol, PGE_2, 5-HT and LTD_4, although they were less potent than the β-agonist salbutamol (94,95). Bronchodilator activity has been demonstrated *in vivo* in models of respiratory distress, and may occur at doses lacking cardiovascular side effects (95,96). In asthmatic patients, orally administered cromakalim was reported to protect against nocturnal bronchoconstriction (97). Mechanistic studies indicate that, unlike the β-agonists, KCAs do not open the CTX-sensitive, high conductance Ca^{++}-activated K^+ channels (98,99). Electrophysiological evidence has indicated that BRL 38227 opens a glibenclamide-sensitive K_{ATP} channel in rabbit and bovine tracheal smooth muscle (17). The relaxant properties of KCAs are not considered to be the result of an effect on cyclic nucleotides (99), although an influence upon i[Ca^{++}] stores may be important (100). Although cromakalim inhibited cholinergic nerve mediated bronchospasm both *in vivo* and *in vitro* (101,102), KCAs displayed greater potency in inhibiting nonadrenergic, noncholinergic nerve mediated constrictor responses (101,102). These latter findings have important clinical consequences, since KCAs may inhibit the release of sensory neuropeptides which may contribute to the inflammatory reaction in asthmatic airways

(103). Thus KCAs may possess antiinflammatory activity in addition to being bronchodilators.

Gastrointestinal smooth muscle - The ability of KCAs to act as gastrointestinal smooth muscle relaxants has been documented (1). Recent evidence suggests that K_{ATP} channels may be implicated in the actions of KCAs, since glibenclamide inhibits relaxation and hyperpolarization in a number of gastrointestinal preparations (104-106). In the guinea pig taenia caeci, **13** was about eight times more active than cromakalim as a relaxant (105). Thus the gastrointestinal relaxant properties of KCAs may find application in the treatment of irritable bowel syndrome.

Detrusor and Ureter smooth muscle - The K^+ channel opening property of KCAs has been confirmed in rat detrusor smooth muscle (107). BRL 38227, pinacidil and **5** were about eightfold more selective as relaxants in vascular tissue than in detrusor muscle. KCAs inhibited the increased detrusor pressure in an *in vivo* model of detrusor instability (108). The clinical significance of these data is unclear as pinacidil failed to improve bladder function in patients with bladder outflow obstruction (109). The selectivity of **5** for ureter smooth muscle and the potential application has been discussed above.

Uterine smooth muscle - Although cromakalim produced minimal hyperpolarization and no change in $^{86}Rb^+$ efflux in uterine smooth muscle (1), further *in vitro* work indicated that the relaxant property of KCAs is related to the opening of K^+ channels (110). This was confirmed *in vivo*, where cromakalim and **21** relaxed the uterus in both non-pregnant and pregnant rats in a glibenclamide-sensitive manner, although blood pressure was reduced at the concentrations used (110). Tolerance developed to the uterine relaxant (111) and hypotensive (112) effects of cromakalim, an observation not previously reported for KCAs. Nevertheless, the uterine relaxant effects of KCAs may prove clinically useful in premature labor and dysmenorrhoea.

CNS - In rat substantia nigra, KCAs enhanced $^{86}Rb^+$ efflux and decreased ^3H-GABA release in a sulfonylurea-sensitive manner, suggesting an association between K_{ATP} channels and reduced transmitter release (113). Surprisingly, the rank order of potency of KCAs differed from other tissues (113). K_{ATP} channels in substantia nigra may play a functional role in seizure control (114). Intracerebroventricular administration of KCAs attenuated seizures in genetically epileptic rats (115) and those induced by MCDP (116). These data support a potential use for selective KCAs in the prophylactic treatment of epilepsy. The description of **12** as an antidepressant indicates an additional potential application in CNS disorders (37).

Other tissues - KCAs were reported to open K_{ATP} channels in the pancreas (1). With the exception of diazoxide, this was observed at concentrations in excess of those required for vasorelaxation (117). This suggests that disturbances in carbohydrate metabolism should not be evident in the clinical situation. Interestingly, **9** appeared to act as a K^+ channel blocker in this tissue (117). KCAs have also been reported to open K_{ATP} channels (118) and also glibenclamide-sensitive, ATP-insensitive K^+ channels in skeletal muscle (20). A beneficial role for KCAs in peripheral vascular disease or in conditions such as hypokalaemic periodic paralysis requires further study (119).

Conclusion - KCAs are under development for hypertension, angina and asthma. In hypertensive patients, the ability of pinacidil to produce regression in left ventricular hypertrophy (120), or reduction in plasma cholesterol and triglycerides (121), augurs well for the use of KCAs in hypertension. In addition to the disorders outlined above, KCAs are under investigation for use in glaucoma (122) and impotence (123). The future probably lies in the design and synthesis of tissue selective KCAs for the range of disorders discussed herein, coupled with extensive clinical trials in order that the full potential of KCAs can be assessed.

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Chapter 9. Endogenous Vasoactive Peptides

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Introduction - There is continuing interest in the role of endogenous vasoactive peptides in the regulation of organ function (1). It is now becoming increasingly clear that many of these vasodilator and vasoconstrictor peptide mediators act in concert to control vascular tone and growth (2,3). The endothelium-derived vasoconstrictor peptide endothelin (ET) is thus known to interact with the renin-angiotensin system and with several other vasoactive agents including the atrial natriuretic peptides (ANP), eicosanoids, and endothelium-derived relaxing factor (EDRF) (4-6). These interactions are clearly important in blood pressure regulation and fluid distribution.

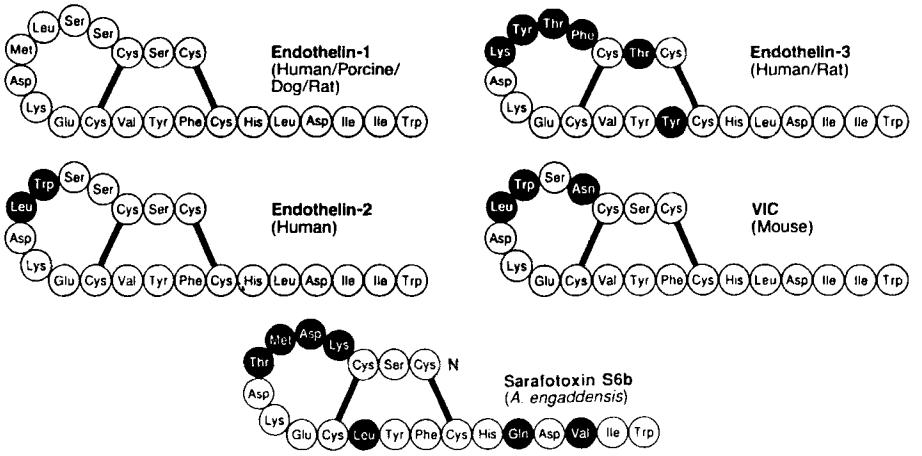
The presence of ET in various regions of the brain indicates a role in neuronal function and central cardiovascular control (7). ET also appears to interact with several other hormonal vasoactive neuropeptides such as neuropeptide Y (NPY), substance P (SP) and arginine-vasopressin (AVP) (8-10).

This chapter will focus on the ET peptide family since there have been many important discoveries in the last year. In addition, key advances in the research ongoing with several other vasoactive peptides will be briefly reviewed.

ENDOTHELIN

Introduction - It has been almost three years since the initial report by Yanagisawa and co-workers describing the isolation and characterization of the vasoconstrictor peptide endothelin-1 (ET-1) (11). During this time the literature on the actions and possible physiological and pathological roles of the ET peptide family has been expanding rapidly (12-14). The structures of these peptides, endothelin-1, -2 and -3 and the related peptide, vasoactive intestinal contractor (VIC), are shown in Figure 1. The snake-venom toxins, sarafotoxins a-d (S6[a-d]), bear a striking structural similarity to the ET's and elicit similar cardiovascular actions (see S6b in Figure 1) (15).

Figure 1. Amino acid sequences of the endothelin peptide family.
Filled circles: residues different from those in ET-1.



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Cardiorenal Actions - The cardiovascular actions of the ET's have been studied in a range of in vitro and in vivo models and have been reviewed recently (16). The ET's elicit a long-lasting vasoconstriction in almost all arteries and veins (17). Intravenous infusion to normotensive and spontaneously hypertensive rats causes a transient hypotensive effect, followed by a sustained pressor response accompanied by a reduction in cardiac output (18). The pressor responses of ET-1 were reduced by Ca^{2+} channel blockers and the K^+ channel opener, cromakalim (18). Low concentrations of ET-1 in mammary artery rings potentiated contractions to norepinephrine and serotonin, suggesting that ET may play an important role in acute ischemic disorders associated with platelet activation (19). The cardiovascular responses to VIC, ET-2 and S6b are similar to those of ET-1, eliciting biphasic changes in arterial pressure and increased central venous pressure, cardiac output and pulmonary arterial pressure (20,21). The chronotropic, positive inotropic, and arrhythmogenic effects of ET have been reported (22).

Intravenous infusions of ET-1 to humans (1, 2.5, and 5.0 ng/kg/min) caused increases in mean blood pressure and serum potassium concentration (23). Plasma concentrations of renin, ANP and aldosterone were unchanged. ET specific monoclonal antibodies attenuated ET-induced contraction of rat aortic rings and the pressor effects of ET-1 in pithed rats (24).

Several studies have shown that ET-1 has an important impact on the kidney, causing reduction in renal blood flow and urinary sodium excretion (25,26). In contrast to ET-1, bolus injection of the 39-residue peptide precursor of ET-1, named big-ET, to conscious rats, did not change renal blood flow or renal resistance. Both peptides caused a dose-dependent diuretic and natriuretic response. These effects indicate different mechanisms of action for the two peptides (27). Renal ET receptor density was found to be significantly higher in cyclosporine-A induced nephrotoxicity (28). In DOCA-salt hypertensive rats, ET clearance is reduced, resulting in a lower renal blood flow compared with normotensive controls (29). ET stimulates ANP release in a conscious rat model (30). In addition, ANP has been shown to block the pressor action of ET (31).

Recent experiments have shown that ET elicits a sustained vasoconstriction and increase in hepatic glucose output in the liver (32).

Mitogenic actions - ET-1 is a potent mitogen in fibroblasts and rat aortic smooth muscle cells and has been implicated in the pathophysiology of atherosclerosis (33,34). ET-1 has also been shown to stimulate mitogenesis in glial cells in the rat brain, and may play a role in wound healing after neuronal injury (35). The mitogenic activity of ET-1 and ET-3 on vascular smooth muscle is inhibited by atriopeptin III (ANP 5-28) (36). The broad spectrum neuropeptide antagonist [D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-Substance P has been reported as an ET/VIC antagonist blocking the binding of ^{125}I-ET-1 (IC_{50} = 40 uM), and its Ca^{2+} mobilizing and mitogenic effects in mouse 3T3 cells (37).

Bronchopulmonary Effects - The effects of ET-1 on the bronchopulmonary system have been reviewed recently (38). A significant inhibitory effect of the platelet activating factor antagonist, BN 52021 (ginkgolide B) or specific thromboxane receptor antagonists on the bronchopulmonary response induced by ET-1 has been reported, supporting a role for the involvement of cyclo-oxygenase products in its effects (39,40). The contractile activity of ET-2 and -3 on human isolated bronchus is less than that of ET-1 (41).

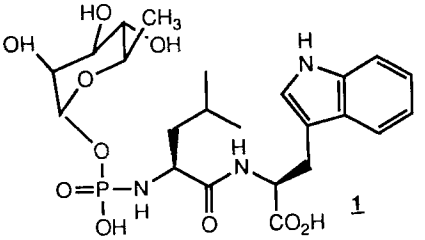
Production and Distribution of ET-1 and Related Peptides - ET-1 is derived from a 203 amino-acid peptide precursor preproendothelin that is cleaved after translation, by endopeptidases specific for the paired dibasic residues to form proendothelin or big ET (11). ET-1 mRNA is widely expressed in rat, porcine, guinea pig and human tissues (42,43). The distribution of big-ET and immunoreactive (i.r.) ET-1 has been compared in porcine tissues (44). The concentration of i.r.-big ET was highest in the aortic intima and lung, while the highest concentration of i.r. ET-1 was found in the kidney inner medulla (44). These data indicate that the biosynthetic path of ET may vary according to the tissue. In addition to endothelial cells, ET-1 is produced by mesangial, kidney, and epithelial cells and also by various human cancer cell lines and human macrophages (45-49). ET gene transcription occurs in a variety of functional regions in the human brain, especially the hypothalmus (50,51). Evidence for transcription and expression of the ET-3 gene in the human placenta and the ET-2 gene in human tumor cells has recently been reported (52,53).

The expression of the preproendothelin gene in cultured cells is stimulated by thrombin, TGF-ß, epinephrine, AVP, phorbol esters and the calcium ionophore A23187 (10,54). Other conditions that cause its release include increased shear stress, hypoxia, oxyhemoglobin, elevated glucose levels and endogenous digitalis-like factor (55-59). In cultured anterior pituitary cells, insulin-like growth factor-1 stimulates ET-3 production (60). In the intact circulation, thrombin and A23187 have been demonstrated to enhance ET-1 release, while EDRF inhibits its production (61,62).

Mechanism of Action - The mechanism of ET action has been studied intensively over the last year. Although extracellular calcium appears to be important, direct activation of the Ca^{2+} voltage-sensitive channels does not seem to be involved since most reports indicate that ET-induced contractions are either not affected or only partially inhibited by L-type calcium channel antagonists (63-65). ET-1 binds to its G-protein coupled receptor to activate phospholipase C, resulting in increased formation of inositol tris and bisphosphates and 1,2-diacylglycerol, with subsequent stimulation of protein kinase C (66-69). The involvement of other phospholipases has also been suggested, and a preliminary model of the signal transduction pathways has been proposed (69). The initial transient vasodilator action of the ET's has been attributed to the release of PGI_2 and/or EDRF (2).

Receptor Studies - One of the most exciting advances in ET research in the last year has been the cloning and expression of two distinct receptor subtypes (70,71). Both are G-protein coupled and belong to the rhodopsin family, with seven transmembrane domains. One is highly specific for ET-1 (MW=48.5 KDa) and is located in the periphery and CNS, and has been suggested to be the vascular smooth muscle (VSM) type (70,72). The other is a "non-selective" subtype that binds ET-1, 2, and -3 with similar affinity (71,73). It is not located in VSM, but is found in endothelial cells and in many rat tissues including brain, kidney and lung (73). There is further evidence for an ET-3 specific receptor subtype that is located primarily in brain and in endothelial cells (74,75). The distribution of the endothelin isopeptide binding sites in porcine tissues has been described (76).

Endothelin-Converting Enzyme - The biosynthetic pathway of ET-1 has been proposed to involve the cleavage of the 39-amino acid propeptide, big ET, via a putative endothelin-converting enzyme (ECE). Much effort has been expended over the last year to identify ECE. Initial reports from endothelial cell cultures implicated the involvement of an aspartic proteinase (77,78). Specifically, pepsin and cathepsin D were shown to cleave big ET to ET in vitro. However cathepsin D causes further rapid degradation of ET-1, and thus it seems unlikely to be involved in its formation in vivo (79,80). Human cathepsin E, a closely related aspartic proteinase, has been shown to specifically cleave big ET at the Trp^{21}-Val^{22} bond in vitro to produce ET-1 with no further degradation (80). There are no reports of studies with specific human cathepsin E inhibitors in vivo to verify the significance of these data. Other reports of attempted isolation of ECE from cultured endothelial cell and vascular smooth muscle have implicated a metalloprotease (81-84). In addition, relatively high concentrations of phosphoramidon (1) have been shown to block the pressor response of big ET in vitro and in vivo (85-87). Polymorphonuclear leukocytes also effect the conversion of big ET to ET that is inhibited by (1) but not by inhibitors of serine, cysteine, aspartate or leucine specific proteases (88). A further complication is the suggestion that the endothelin isopeptides may be processed by different pathways (81).



1

Metabolism - The kinetic properties and mode of hydrolysis of the ET's by neutral endopeptidase 24:11 (enkephalinase)involves cleavage at the Ser^5-Leu^6 bond, followed by rapid cleavage between $Asp^{18}$and Ile^{19} (89).

Structure-activity Relationships - The discovery of selective ET receptor antagonists will facilitate identification of the physiological and pathological roles of the various ET isopeptides. The 3-dimensional structure of ET and various analogs have continued to be studied by NMR and circular dichroism (90-92). The disulfide linkages and resulting conformational properties are important for binding and functional activity in porcine and rat aorta (93,94). In other studies, reduction and carboxymethylation of the cysteine residues caused a complete loss of agonist activity in rat

isolated perfused mesentery and the tetra-alanyl analog ET-1[Ala-1,3,11,15] was also functionally inactive in the rat mesenteric bed (95,96). However, the structural requirements for binding to rat cerebellum do not require the presence of the disulfide linkages as illustrated by the equipotent binding of the tetra-alanyl substituted analog ET-1[Ala-1,3,11,15] and ET-1 (97). These results in various tissues may be due to a different distribution of receptor subtypes (96).

Other residues reported to be important for vasoconstrictor activity in the rat thoracic aorta and isolated mesentery include Trp^{21}, the loop region residues 5-9, Lys^{9} and Tyr^{13} (95). Receptor binding results in cultured rat smooth muscle cells revealed that ET[1-23] and ET[1-26] were equipotent with ET-1, although functional studies demonstrated that these C-terminal elongated peptides were weaker agonists (98).

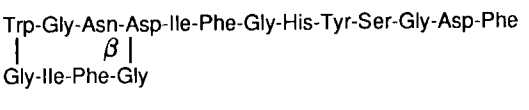
The $[Ca^{2+}]_i$ response in the fura-2 loaded VSM cell line A7 was measured for the C-terminal nonapeptide and N-terminal octapeptide: both showed partial agonist activity (99). Several reports describing receptor binding and functional activities of the C-terminal hexapeptide region have appeared (100-102) in a variety of tissue preparations. Functional studies in the guinea pig bronchus, which is particularly sensitive to the C-terminal region, indicate that the Trp^{21}, Asp^{18}, His^{16}, and Leu^{17} residues are important for biological activity (101). D-amino acid substitutions at His^{16} and Ile^{20} resulted in increased binding affinity in rabbit aorta and pulmonary artery and in rat heart although no functional activity was reported (102). In a binding assay in rabbit cardiac tissue, ET[1-20], ET[1-15]-NH_2 or the C-terminal hexapeptide, ET[16-21], were completely inactive (103). In this report other analogs indicated the importance of the disulfide linkage 3-11, Phe^{14}, His^{16}, and/or Asp^{18} for binding. Formylation of the Trp^{21} or replacement of the Leu^{7} for Met^{7} does not affect binding, while C-terminal amidation completely abolishes it (103).

Binding and vasoconstrictor activities of a range of monocyclic analogs, ET-1[$Ala^{3,11}$, Nle^{7}] containing mono Ala substitutions has shown that Glu^{10}, Phe^{14}, Leu^{17} and Asp^{18} may be important for agonist activity, while Asp^{8}, Tyr^{13}, Ile^{20} and Trp^{21} are important for binding (104). The residues Ser^{2}, Val^{12}, His^{16} and Ile^{19} were less important for binding or agonist activity. The functional activities of ET-1[12-Pro] and ET-1[16-Phe] have been compared in isolated vascular and cardiac tissues and the latter compound is a more potent agonist than ET itself (105). The binding affinity of various monocyclic fragments of ET-1 in rabbit pulmonary artery and aorta have indicated that the loop region 3-11 does not bind at concentrations up to 100 uM. Analogs without the 3-11 region bind with uM affinity but show no functional activity up to 30 uM (106).

A number of analogs were synthesized to investigate their effect on the pulmonary vasodilator response when compared to ET-1 (107). Intralobar injections of ET[16-21], big ET-1[22-39] and ET-1[1,3,11,15-Ala] to the intact cat did not alter arterial blood pressure while ET-1, ET[1-15] and big ET caused a decrease in lobar arterial blood pressure (107). These results indicate that only the intact amino terminus and intra-chain disulfide bridges are necessary for pulmonary vasodilation.

ATRIAL NATRIURETIC PEPTIDE

Role of ANP - Elevated plasma levels of the vasodilator, atrial natriuretic peptide (ANP), have been found in several cardiovascular disorders associated with intravascular volume overload and/or increased venous pressures (108,109). The significance of these findings has been difficult to explain due to the lack of a specific ANP antagonist. Recently, a functional antagonist of ANP known as anantin (**2**) was isolated from *Streptomyces coerulescens* (110).



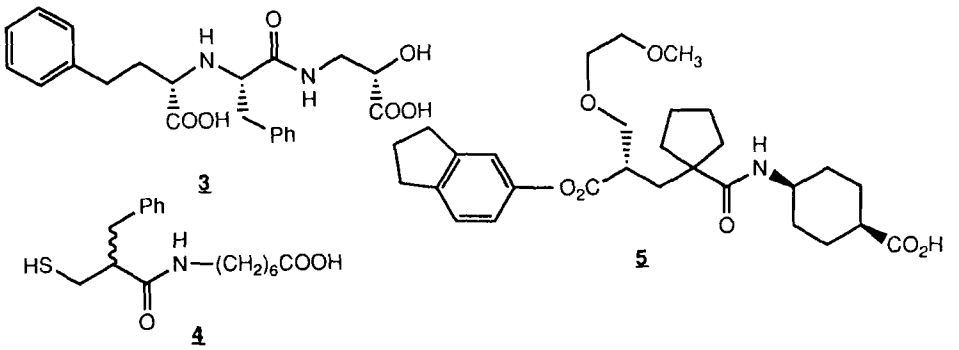
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There have been many studies of ANP infusions to animals and humans to study its role in cardiovascular disorders (109,111,112). Infusion of anti-ANP antibodies to a rat model of chronic

congestive heart failure (CHF) significantly increased right atrial pressure, left ventricular end diastolic pressure and systemic vascular resistance (113). These studies provide some evidence for the role of ANP in chronic CHF. The possible role of ANP in hypertension was studied using transgenic mice that have elevated levels of ANP in the systemic circulation (114). The transgenic animals had significantly lower mean arterial blood pressure (75.5 ± 0.9 mmHg) compared with non-transgenic siblings (103.9 ± 2.0 mmHg) (114). Other physiological parameters, including heart rate, plasma and urinary electrolytes, H_2O intake, and urine volume, were unchanged. A recent study has shown that ANP inhibits hypertrophy of VSM cells that may serve as a compensatory mechanism to hypertensive vascular changes, in addition to vasorelaxation and natriuresis (115).

Receptor Studies - The molecular biology of the ANP receptor subtypes has been reviewed recently (116). The amino acid sequence of human ANP clearance receptor deduced from the nucleotide sequence of cDNA clones from human placental and kidney cDNA libraries was reported to be highly homologous to the bovine C-receptor sequence previously described (117). The first reported primary structure of the rat guanyl cyclase A ANP receptor has been disclosed (118).

Neutral Endopeptidase Inhibitors - The zinc metalloprotease neutral endopeptidase 24:11 (NEP) is known to be involved in the degradation of a variety of peptides, including ANP (119). Further preclinical and clinical studies with NEP inhibitors in models of hypertension and heart failure have appeared, and the subject has been reviewed recently (120). The effects of the NEP inhibitor SCH 39,370 (3) in a rat model of severe heart failure indicated no change in renal function or systolic blood pressure. Plasma ANP levels and cyclic GMP urinary excretion were clearly increased over controls (121). The effects of SQ 29,072 (4) have been studied in conscious SHR and DOCA/salt hypertensive rats, and it causes a potentiation of endogenous ANP by NEP inhibition (122). Administration of UK 79300 (candoxatril) (5) to normal human subjects results in a statistically significant enhancement of plasma ANP, a clear natriuresis, and an increase in cyclic GMP, with suppression of the renin-angiotensin-aldosterone system (123).



NEUROPEPTIDE Y

NPY Antagonists - The 36-residue, C-terminally amidated peptide NPY, occurs ubiquitously in the central nervous system and in peripheral nerves (124,125). The potential involvement of this vasoconstrictor in the regulation of cardiac function may be elucidated by the study of several newly discovered NPY antagonists. Two related side-chain modified NPY fragments (6) and (7) were reported to possess NPY antagonist activity (126).

NPY: Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y-NH_2

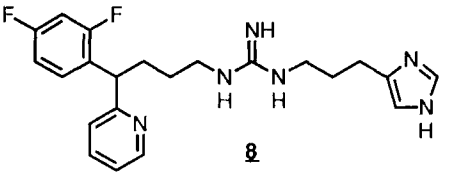
6: Ac - Y-I-N-L-I-T-R-Q-R-Y-NH_2
2,6-Cl_2Bzl

7: Ac-Y-I-N-L-I-T-R-Q-R-Y-NH_2
2,6-Cl_2Bzl 2,6-Cl_2Bzl

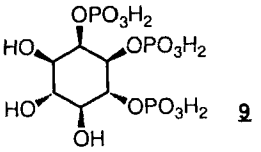
The C-terminal fragment NPY[18-36] was reported to be a competitive, selective antagonist of NPY in rat cardiac ventricular membranes (K_i = 140 nM) (127). The acetylated analog behaved as

an agonist of NPY (127). One of the first non-peptide NPY antagonists, He-90481 (8) was recently disclosed. Pharmacological use of this compound will be limited by its low affinity and H_2 agonist effects (128).

The antagonist activity of D-myo-inositol-1,2,6,-triphosphate (PP56, (9)) on NPY-induced contraction of the guinea pig basilar artery has been reported at concentrations of 10^{-8}-10^{-6} M (129). At higher concentrations (9) appears to modify the responses of histamine and acetylcholine.



8



9

$RNH(CH_2)_6NH(CH_2)_2S\text{--}S(CH_2)_2NH(CH_2)_6NHR$

$R = 2\text{-}CH_3O\text{-}C_6H_4CH_2\text{-}$

10

The tetramine disulfide antagonist of α-adrenoreceptors, benextramine (10) produces a long-lasting antagonism of NPY's pressor activity. It irreversibly inhibited binding to a sub-population of NPY receptors in rat brain membranes (130).

Receptor Studies - Solubilization and characterization of NPY receptors from rabbit kidney have led to the identification of a 100 KDa peptide representing covalently labelled receptor complex (131). At least one NPY receptor subtype belongs to the class of G-protein coupled receptors, but the exact signalling pathways are not completely understood. A common 65 KDa cell surface receptor for pancreatic polypeptide, NPY and peptide YY was reported recently (132). Reasonably selective ligands for peripheral NPY receptor subtypes, Y_1 and Y_2, have been reported (Table 1) (133-136). The classification of receptor subtypes as postsynaptic/presynaptic is somewhat controversial (137).

Table 1. NPY Receptor Subtypes

Subtype	Activation	Selective agonist	Selective antagonist	Activity
Y_1	entire NPY molecule	NPY[Leu^{31}, Pro^{34}]		postsynaptic
Y_2	Long C-terminal fragments or full length NPY analogs	NPY[13-36]	NPY[18-36]	presynaptic

CALCITONIN-GENE RELATED PEPTIDE/AMYLIN

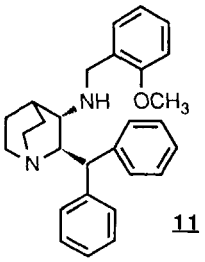
The localization and effects of calcitonin-gene related peptide (CGRP) have been reviewed recently (138). It is a potent vasodilator of arterial smooth muscle existing in two closely related forms, α and ß-CGRP (138). The role of CGRP in hypertension, heart failure and other cardiovascular diseases is currently not known. CGRP has been studied in patients with angina pectoris and found to improve ischemic threshold and maximum cardiac workload (139). It has been administered to patients with severe complicated Raynaud's phenomenom and found to be an effective peripheral vasodilator (140). In addition to its vasodilatory actions, CGRP is a potent regulator of glycogen metabolism in rat skeletal muscle, and may be involved in the pathogenesis of Type II diabetes (141). Human α-CGRP [8-37] has been reported as an effective antagonist of the cardiovascular actions of α-CGRP in vivo (142). Agonist activity has been reported for several N-terminal containing CGRP fragments (143).

Amylin amide, a 37-amino acid peptide, has considerable homology with CGRP(46%) and is the major component of amyloid deposits found in the ß-cells of the islets of Langerhans of Type II diabetes patients (144). This peptide stimulated a dose-dependent increase in blood flow after intradermal injection into rabbit skin (145). However amylin is about 100x less potent as a vasodilator than CGRP (145). Both amylin and CGRP have been reported to cause insulin

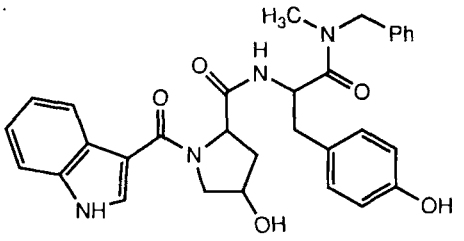
resistance in vivo (146). There is no sequence conservation over residues 20-29 in amylin and CGRP, and this is the region thought to be important for the formation of amyloid deposits (147).

TACHYKININS

The vasodilator Substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), a member of the tachykinin peptide family, has a broad spectrum of biological activities in addition to its possible role as a neurotransmitter in the CNS (148). There has been considerable interest in the possibility that SP antagonists might be useful as novel analgesics or neuroleptic drugs. In the periphery, SP antagonists may be of use as antiinflammatory or hypotensive agents. The first non-peptide SP antagonists have recently been reported, and should be useful tools for elucidating the role of SP (148,149). In the CNS, CP-96,345 (**11**) is a potent inhibitor of [^{3}H]-SP binding to bovine caudate membranes (IC_{50}=3.4 ± 0.8nM) (149,150). Other closely related quinuclidine derivatives have been disclosed as SP antagonists (151). New dipeptide tachykinin antagonists, such as compound (**12**) have been shown to inhibit SP induced bronchoconstriction after intratracheal administration to guinea pigs (152).



11



12

Conclusion - Vasoactive peptides and their receptors participate through a variety of mechanisms in the regulation of vascular tone and cardiovascular function. The search for specific non-peptide receptor antagonists and processing enzyme inhibitors of these vasoactive substances is of great importance to improve our understanding of their role in the pathophysiology of human diseases.

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Chapter 10. Emerging Drug Discovery Targets in Thrombosis and Coagulation

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Introduction - In the United States alone, approximately 1.5 million persons per year experience a myocardial infarction (1). The etiology of myocardial infarction has become much clearer in the past decade with the seminal observation that thrombosis at the site of previous vascular atherosclerotic narrowing is an important factor in acute occlusion of coronary blood vessels (2). This has led to an explosion of activity in attempts to restore blood flow of occluded coronary arteries with thrombolytic agents. However, despite the ability of thrombolytic therapy to restore blood flow in 60-70% of patients with an occluded coronary artery, many problems remain. For instance, not all patients reperfuse in response to thrombolytics and of those who do undergo successful coronary thrombolysis, an unacceptable fraction will experience acute reocclusion. The factors related to initial thrombosis and rethrombosis following thrombolysis are complex but appear to involve the interaction of blood platelets, the coagulation cascade and the endogenous fibrinolytic capacity of the patient. Thus, platelet aggregation, blood coagulation and endogenous fibrinolysis represent viable drug discovery targets as a means to improve the morbidity and mortality associated with myocardial infarction. Therefore, this chapter will be divided into 3 major sections dealing with new drug discovery targets related to 1) inhibition of platelet aggregation, 2) interruption of the coagulation cascade and 3) modulation of the endogenous fibrinolytic system.

INHIBITION OF PLATELET AGGREGATION

Blood platelets play a key role in homeostasis and thrombosis. Over the years numerous approaches have been utilized in attempts to interrupt the process of platelet activation and subsequent aggregation in thrombotic diseases. Many of these approaches were directed against individual mediators of platelet aggregation such as thromboxane A_2 (3), serotonin (4) and platelet activating factor (5) with highly specific receptor antagonists or enzyme inhibitors. However, these approaches left intact other mediators of platelet aggregation such as ADP, collagen, epinephrine and/or thrombin. Since the exact nature of the mediator of platelet aggregation in pathophysiological conditions is unknown, attempts have been made to interrupt the process independent of which agonists are responsible for the activation. The first such agent was prostacyclin (PGI_2), which elevates platelet cyclic AMP, (6) resulting in inhibition of platelet function in response to all known physiological platelet agonists. However, PGI_2 also elevates smooth muscle cell cyclic AMP which leads to unacceptable vasodilation at antithrombotic doses (7). The inability to separate the broad spectrum anti-platelet effects of PGI_2 from hemodynamic changes has led to a search for agents with the same anti-platelet profile but without the associated hemodynamic changes. A number of these agents have been discovered, and all rely on interruption of the final common pathway of platelet aggregation, which is binding of plasma fibrinogen (8). Upon platelet activation with the above mentioned mediators of platelet aggregation, the platelet receptor for fibrinogen, glycoprotein IIb/IIIa (GPIIb/IIIa) is expressed. The tripeptide arginine-glycine-aspartic acid (RGD), which occurs twice in the Aα chain of fibrinogen, is believed to mediate at least in part binding of fibrinogen to the GPIIb/IIIa complex (9,10). Thus, binding of fibrinogen to activated platelets is a key event in platelet-platelet interaction and plays a major role in thrombus formation.

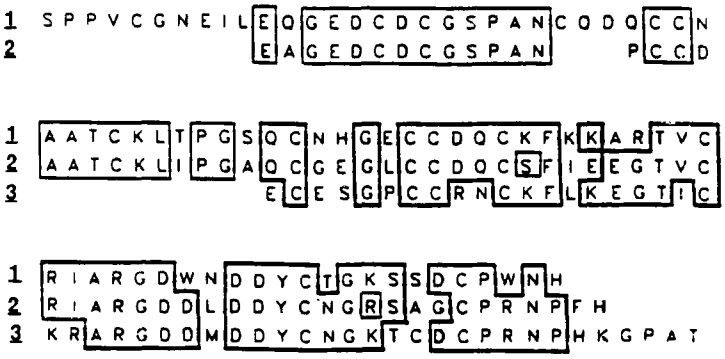
Pharmacological agents have been developed which interrupt the interaction of plasma fibrinogen with the platelet GPIIb/IIIa complex. These agents are either monoclonal antibodies directed against GPIIb/IIIa, or are peptide or non-peptide mimetics of RGD which block the binding of fibrinogen to the

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GPIIb/IIIa complex. These agents are devoid of hemodynamic effects and appear to be important new antithrombotic compounds, many of which are under or awaiting clinical evaluation.

Monoclonal Antibodies Directed Against The Platelet Membrane GPIIb/IIIa Receptor - A murine monoclonal antibody (7E3) to the platelet GPIIb/IIIa complex was developed and subsequently demonstrated to produce an antithrombotic effect in dogs and monkeys when infused intravenously (11,12). The same antibody was then assessed in combination with tissue-type plasminogen activator (tPA) and was shown to accelerate thrombolysis in dogs and prevent reocclusion (13,14). These actions to improve the efficiency of thrombolysis are related to the activation of platelets by thrombolytics (15-17) and the high degree of thrombogenicity which exists in diseased coronary arteries leading to reocclusive events. The 7E3 antibody, which is a $F(ab')_2$ fragment, has been evaluated in preliminary clinical studies and has led to impressive suppression of ex vivo platelet aggregation and prolongation of the bleeding time (18). However, once the antibody binds to the GPIIb/IIIa receptor on the platelet surface, the inhibition of platelet function is essentially irreversible due to the tight binding affinity of the antibody for the receptor (18). Since platelets have a life span of 7-10 days, new platelets which enter the circulation restore the hemostatic state of the patient. Thus, once the antibody has been administered and platelet function inhibited, it may not possible to readily reverse the effect if so desired. In the thrombolytic arena, where bleeding is a serious concern due to the potential of fatal or disabling intracranial hemorrhage, this approach will require careful safety evaluation before it is accepted into medical practice. Due to lack of acute reversibility with the antibody directed approach, other means to interrupt platelet binding of fibrinogen via GPIIb/IIIa have been investigated which allow for rapid restoration of platelet function following termination of intravenous infusion of the agent.

Viper Venom Peptides As Functional Antagonists Of The Platelet GPIIb/IIIa Receptor - As mentioned previously, platelet binding of plasma fibrinogen occurs through a specific RGD sequence in the Aα chain of fibrinogen. Thus, agents which contain an RGD sequence should be able to effectively compete with fibrinogen binding to the GPIIb/IIIa complex on the platelet surface. On this basis, viper venom peptides which contain RGD sequences have been identified and evaluated extensively as antithrombotic agents. A whole family of viper venom peptides have been extracted from crude venom and sequenced. Of these the most notable are bitistatin (1) from the venom of Bitis arientans (19), trigramin (2) from the venom of Trimeresurus gramineus (20,21), and echistatin (3) from the venom of Echis carinatus (22). The single letter amino acid sequences of bitistatin, trigramin and echistatin demonstrate considerable homology with the RGD sequence being the most important for maintaining the biological activity directed against platelet aggregation.



Trigramin is a 72-amino acid peptide which inhibits ADP-induced platelet aggregation with an IC_{50} value of 1.3×10^{-7} M (20) and inhibits platelet hemostatic plug formation in vivo with a potency of almost 3 orders of magnitude greater than that achieved with authentic RGDS itself (21).

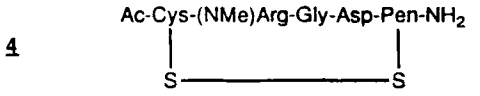
Echistatin and bitistatin are 49- and 83-amino acid containing inhibitors of platelet aggregation with IC_{50} values against human platelet aggregation in platelet rich plasma induced by ADP of 101.3 ± 4.3

and 237.2 ± 13.5 nM, respectively (19,23). Echistatin and bitistatin inhibit platelet-dependent coronary thrombus formation in the Folts model of partially obstructed coronary arteries (19,23) with dose-dependent elevation of the bleeding time and inhibition of ex vivo platelet aggregation. In a canine model of coronary thrombosis elicited by electrolytic injury to the endothelial surface of the blood vessel, echistatin (24) and bitistatin (25) are effective in improving tPA-induced thrombolysis with a greater incidence of reperfusion and a decrease in the time to reperfusion; these effects were significantly different from control animals given tPA alone. Furthermore, in these same studies, echistatin and bitistatin were extremely effective in preventing platelet-dependent acute reocclusion which occurs after successful lysis in a high percentage of control animals, even in the presence of high-dose heparin. Thus, these studies demonstrate the importance of inhibiting platelet function concomitant and subsequent to coronary artery thrombolysis, such that reperfusion parameters can be improved and acute reocclusion avoided.

The viper venom inhibitors of platelet GPIIb/IIIa are effective pharmacological tools in which the anti-platelet activity of this approach can be evaluated experimentally (24,25). However, due to the large size of these inhibitors which would preclude efficient production of material, and the possibility of immune responses being elicited by foreign proteins, these agents are not prime clinical candidates (23,25). They do serve a useful role as templates for smaller peptide and non-peptide mimetics which may be useful as pharmacological drugs if they maintain the biological specificity and activity of the larger natural venom peptides.

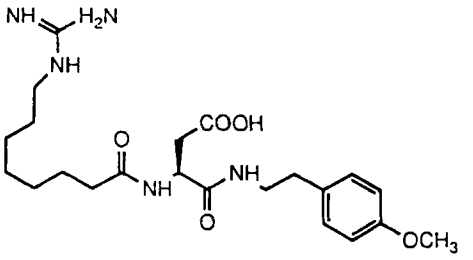
Small Peptide And Peptide Mimetics Of Viper Venoms Directed Against GPIIb/IIIa - In an effort to develop an antithrombotic against platelet GPIIb/IIIa, small peptide inhibitors containing an RGD sequence have been synthesized. Authentic RGDS (S=Serine) is in itself antithrombotic but extremely high concentrations must be infused locally in order to demonstrate an effect in vivo (26). This is presumably due to the low affinity of native RGDS for the GPIIb/IIIa receptor complex. RGDY (Y=O-methyltyrosine) has been evaluated in a canine model of thrombosis/thrombolysis and was partially effective at a dose (4 mg/kg/min) which was 1000 times greater than required with snake venom peptides such as bitistatin to achieve similar endpoints (27). Thus, the conformation and/or searching binding interactions are probably extremely important in determining the potency of these agents in vivo, as RGDS and RGDY are impotent compared to viper venom peptides which also contain the RGD sequence, but demonstrate high potency. Other linear peptides have been synthesized which inhibit fibrinogen binding to platelets such as GPRP and mimetics of the fibrinogen γ chain such as LGGAKQAGDV (28).

In an effort to design more potent small inhibitors of GPIIb/IIIa, cyclical peptides have been synthesized with the hope that these compounds will have greater activity than their linear RGD-containing predecessors. The first of these compounds is SKF 106760 (4) which is a tetra-peptide cyclized with penicillamine to produce a ring structure (29). This compound displayed potent inhibiton of platelet-dependent thrombus formation in stenosed coronary arteries (effective dose 1-3 mg/kg, i.v.) and accelerated streptokinase (SK)-induced thrombolysis from 34 ± 8 to 14 ± 3 min. Furthermore, SKF 106760 reduced acute platelet-dependent reocclusion following successful lysis from 83% in the control group treated only with SK to 14% in the SKF 106760 drug-treated group (29).



Peptide mimetics of RGD-containing peptides have also been synthesized in an attempt to gain greater plasma stability and oral activity. In the prototypic compound of this class, the RG dipeptide segment was replaced with a guanidino-octanoate moiety and the terminal amino acid was replaced by phenethylamine. These substitutions led to SC-47643 (5), a compound that contains only one natural amino acid, aspartate (30). Because this compound has no natural peptide bonds and has biological activity almost identical to known RGD-containing peptides, SC-47643 is referred to as a peptide mimetic.

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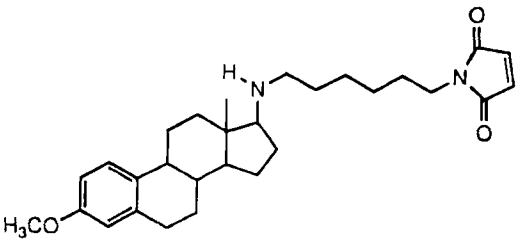


SC-47643 inhibited platelet aggregation in response to a large array of platelet agonists in both washed and platelet-rich human plasma (30). SC-47643 also inhibited fibrinogen binding to activated platelets with an IC_{50} of 1.0 X 10^{-5} M. These biological actions are consistent with other known RGD-containing peptides which have previously been characterized as fibrinogen receptor antagonists. In vivo, SC-47643 was effective in preventing collagen-induced thrombocytopenia in rats (ED_{50} = 0.07 mg/kg, $t_{1/2}$ 36 min) and in dogs blocked collagen-induced ex vivo platelet aggregatory responses 50% following bolus injection of 1.7 mg/kg, i.v. Steady state infusions (ED_{50} = 0.03 mg/kg/min) had no effect on blood pressure, heart rate or platelet count (29). These data demonstrate that SC-47643, a peptide mimetic of the natural fibrinogen RGD binding sequence, is capable of blocking platelet-fibrinogen interactions and platelet aggregation induced by multiple mediators.

Phospholipase C Inhibitors - Agonist-receptor interactions on the platelet plasma membrane are responsible for a cascade of events which results in platelet binding of plasma fibrinogen. Since 1984 it has been known that agonist stimulation results in activation of a specific phospholipase C that preferentially cleaves phosphatidylinositol-bis-phosphate, releasing both diacylglycerol and inositol-tris-phosphate (31). Diacylglycerol activates protein kinase C and inositol-tris-phosphate mobilizes calcium from intracellular stores. Specific G proteins are thought to mediate the activation of phospholipase C and, possibly phospholipase A_2 (32). Phospholipase C-induced calcium mobilization results in activation of phospholipase A_2 which releases arachidonic acid from membrane phospholipids resulting in thromboxane A_2 formation, a potent mediator of platelet aggregation and smooth muscle contraction (33). Thus, selective inhibiton of receptor-coupled phospholipase C may be an effective means to interrupt platelet function to a broad array of agonists by shutting down calcium mobilization from intracellular stores which is obligate in platelet activation and subsequent aggregation.

Recently U-73122 (6), an aminosteroid, has been demonstrated to be a potent inhibitor of human platelet aggregation purportedly through inhibition of platelet phospholipase C (34). U-73122 inhibited the production of inositol 1,4,5-trisphosphate and the subsequent rapid increase in cytosolic calcium induced by thrombin or by a thromboxane A_2 mimetic. Inhibition of platelet aggregation was not mediated by an increase in platelet cyclic AMP and the activity of phospholipase A_2 was unaffected. These data are consistent with interruption of phospholipase C either directly or through interactions with specific G proteins which regulate the activity of phospholipase C.

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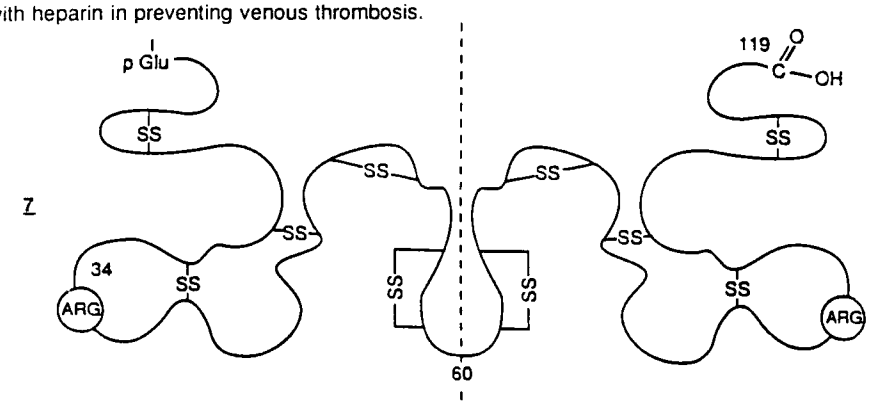


INTERRUPTION OF THE COAGULATION CASCADE

The coagulation cascade is an amplifying system which results in the formation of the active enzyme thrombin which ultimately catalyzes the conversion of plasma fibrinogen to fibrin. Thrombin also catalyzes the conversion of factor XIII to activated factor XIII (XIIIa) which crosslinks fibrin resulting in a stable clot. However, numerous reactions occur in the intrinsic and extrinsic pathways of the blood coagulation cascade before the formation of thrombin, which culminate in the formation of the serine protease factor Xa. This enzyme (factor Xa) then catalyzes the common reaction for both pathways which is the generation of thrombin from prothrombin following the assembly of the prothrombinase complex on an appropriate phospholipid surface. Thus, many potential targets for drug intervention exist in the coagulation cascade. Thrombin inhibition is of extreme interest due to its dual role in promoting coagulation as well as stimulating platelet aggregation. However, interruption of coagulation at an earlier stage, such as factor Xa inhibition, may be an important therapeutic target.

Inhibitors Of Factor Xa - Regulation of aberrant hemostasis is an important clinical concern and has focused primarily on the inhibition of thrombin. Due to the dual role of thrombin to activate platelets as well as convert plasma fibrinogen to fibrin, attempts have been made to interfere with the action of thrombin in the cardiovascular system. Standard, unfractionated heparin is currently used as the anticoagulant of choice in the clinical setting. The mechanism of action of heparin involves the acceleration of antithrombin III (ATIII)-mediated inactivation of several serine proteinases in the coagulation cascade. Fractionated low molecular weight heparins (LMWH) have been shown to preferentially enhance the binding of ATIII to factor Xa resulting in a high anti-factor Xa/anti-thrombin ratio (35). Although LMWH have demonstrated antithrombotic properties in experimental models of thrombosis (36-39) and clinically (40-43), the relative role of factor Xa versus thrombin inhibition remains controversial. An understanding of the role played by these two proteinases in thrombotic disorders is important in the development of future anticoagulants and can best be addressed with highly selective, active-site directed inhibitors.

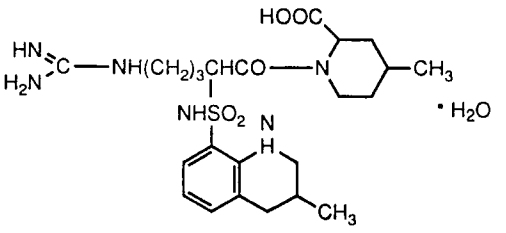
Recently, two highly selective polypeptide inhibitors of factor Xa have been described. The first inhibitor, termed antistasin (ATS), is a 119 amino acid protein (7) which was isolated from salivary gland extracts of the leech Haementeria officinalis (44). ATS is a reversible, slow, tight binding inhibitor of factor Xa with an estimated dissociation constant (Ki) for the enzyme:inhibitor complex of 0.3 nM (45). A second polypeptide inhibitor was isolated from extracts of the tick Ornithidorous moubata (46). Like antistasin, tick anticoagulant peptide (TAP) is a reversible, slow, tight binding inhibitor of factor Xa with an estimated K_i of 0.5 nM (46). TAP is a single chain acidic polypeptide composed of 60 amino acids. ATS and TAP have been shown to be highly selective for factor Xa with no inhibition of thrombin observed for either inhibitor (45,46). Recently the anticoagulant properties of ATS and TAP were compared to heparin in a standard model of thromboplastin-induced clot formation in the rabbit jugular vein (47). This study determined that when ATS, TAP or heparin were prophylactically administered before clot induction, there was significant inhibition of clot mass by all 3 treatments. Recombinant ATS (rATS) and TAP (rTAP) were utilized in this study and it was determined that rATS was approximately 10-fold more potent as an anticoagulant than rTAP. Whereas heparin and rATS elevated APTT clotting time significantly at doses which lowered clot weight, rTAP was effective in reducing clot mass with little or no elevation in APTT clotting time. These data suggest that factor Xa-mediated inhibition of coagulation is as effective as conventional antithrombin treatment with heparin in preventing venous thrombosis.



Thrombin Inhibition With Active Site Inhibitors - Much attention has recently focused on inhibition of thrombin as an emerging means to specifically interrupt the coagulation process. Furthermore, thrombin inhibitors may also have the advantage of suppressing thrombin-induced platelet aggregation and preventing factor XIIIa-mediated fibrin cross-linking. Due to the dual role of coagulation and platelet aggregation in promoting thrombus formation, it seems reasonable to pursue this line of drug development.

Argatroban (MCI-9038) (**8**) is a synthetic analog of arginine which is a competitive thrombin inhibitor (48,49). The IC_{50} value for argatroban in a thrombin clotting time assay with fibrinogen as substrate was 32 nM and K_i value was 19 nM as estimated with chromogenic substrate, S-2238 (50). This compound also inhibits platelet aggregation to thrombin with an IC_{50} value of 9 nM with little or no effect observed on collagen- or ADP-induced aggregation (49). Argatroban has exhibited potent antithrombotic activity in animal models of experimental thrombosis (51-53) and was recently demonstrated to be superior to heparin in prevention of platelet-rich arterial thrombus formation in a rabbit everted femoral artery segment (54). Thus, competitive inhibition of thrombin appears to represent a viable means to interrupting thrombus formation, and acute reversibility may make this approach feasible as a clinical antithrombotic candidate.

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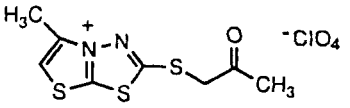
Hirudin is a thrombin-specific inhibitor isolated from the salivary glands of the leech Hirudo medicinalis (55). This agent is a single polypeptide chain protein containing 65 residues (56). Anti-thrombin activity in hirudin is known to follow from formation of a tight, stoichiometric complex between inhibitor and enzyme with a dissociation constant of 20 fM (57). Recently, recombinant hirudin has become available and is comparable in activity to naturally occurring hirudin (58). Further analysis of the recombinant material in pigs indicates that hirudin is superior to conventional heparin in preventing platelet deposition following deep arterial injury of carotid arteries following balloon angioplasty (59, 60).

Another approach to inhibition of the biologic actions of thrombin revolves around the utilization of anti-thrombin chloromethyl ketones. D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) is a potent, irreversible inhibitor of thrombin induced clot formation and platelet aggregation (61). This agent has been assessed in numerous animal models of thrombosis and recently has been demonstrated to interrupt acute platelet-dependent thrombosis on Dacron vascular grafts in baboons (62) and prevent thrombus formation on endovascular stents in baboons (63). The short half-life of this agent requires i.v. infusions in order to demonstrate efficacy in these models of thrombosis.

Factor XIIIa Inhibition - Factor XIII is activated by thrombin to Factor XIIIa which catalyzes fibrin cross-linking with resulting clot stabilization (64). Factor XIIIa catalyzes several covalent crosslinking reactions, which include formation of fibrin γ chain dimers, incorporation of a potent plasmin inhibitor, α-2 antiplasmin, into the α chain of fibrin and the formation of fibrin α chain polymers (65-69). The first two reactions are relatively rapid and are virtually complete within 2 to 5 minutes after the initiation of clot formation, The latter reaction is much slower and may require hours for completion. A pharmacological tool, L-722,151 (**9**), has been utilized *in vivo* to probe the importance of clot stabilization by factor XIIIa in thrombosis and thrombolysis in animal models. Pretreatment with L-722,151, an inhibitor of factor XIIIa, resulted in accelerated thrombolysis in the rabbit (70) and the dog (71), presumably due to inhibition of crosslinking which makes the clot more susceptible to

thrombolysis, a concept previously demonstrated in vitro (72,73). However, administration of L-722,151 15 minutes following thrombus formation, failed to accelerate thrombolysis (71), which indicates that the rapid reactions described previously predominate in the resistance of the clot to lysis. Thus, factor XIIIa represents a viable target; however, the full potential of this approach may only be realized during prophylactic therapy in a patient population at high risk of thrombosis such as that encountered in unstable angina or repeated and predictable episodes of deep vein thrombosis. Recently, in an animal model of deep vein thrombosis, L-722,151 has been shown to enhance thrombolysis with streptokinase and reduce residual thrombus mass significantly compared to non-L-722,151 treated controls (74).

9



Endogenous Anticoagulant Mechanisms - APC is an antithrombotic serine protease which is generated from vitamin K-dependent plasma protein C by the catalytic complex of thrombin and thrombomodulin (75). APC acts by inhibiting thrombin formation by enzymatic cleavage and destruction of coagulation factors Va and VIIIa which provides negative feedback regulation of coagulation (76). Recently recombinant APC was evaluated in a nonhuman primate model of arterial thrombosis and inhibited platelet-dependent thrombus formation onto thrombogenic grafts at plasma levels which did not significantly impair primary hemostasis (77).

MODULATION OF THE ENDOGENOUS FIBRINOLYTIC SYSTEM

The endogenous fibrinolytic system has been implicated in the etiology of cardiovascular disease. Specifically, the lack of a sufficient fibrinolytic capacity has been noted in patient populations with thrombosis due possibly to elevated levels of plasminogen activator inhibitor-1 (PAI-1). PAI-1 is a member of the SERPIN class of proteinase inhibitors and is a specific, fast acting inhibitor of tPA and urokinase (78,79). PAI-1 circulates in plasma normally at low levels and is stored in platelets where it is released upon platelet activation (80,81). PAI-1 could modulate the fibrinolytic pathway by inactivating endogenous plasminogen activators. Another potential modulatory step in the fibrinolytic cascade is α-2 antiplasmin (α2-AP) which is the physiological modulator of plasmin. Plasmin is formed by cleavage of plasma plasminogen by plasminogen activators such as tPA or UK. Thus high levels of α2-AP may inactivate plasmin such that effective fibrinolysis cannot occur. Antibodies have been developed against α2-AP and in experimental models have led to an enhancement of fibrinolysis.

Inhibition Of PAI-1 As A Means To Normalize A Low Fibrinolytic Potential Due To High Circulating Levels Of Inhibitor - PAI-1 has been suggested to be a risk factor for cardiovascular diseases as elevated levels of PAI-1 correlate with venous thrombosis (82), coronary artery disease (83) and recurrent myocardial infarction (84,85). Since inhibitors of PAI-1 have not been described, experimentation has focused on administration of recombinant PAI-1 in animal models of thrombosis or evaluation of transgenic animals with high circulating levels of PAI-1 in order to delineate the role of endogenous plasminogen activators in control of thrombus formation.

When human recombinant PAI-1 was infused to dogs undergoing electrically-induced thrombus formation, the time to complete thrombotic occlusion was accelerated (86). These data seemed to indicate that inhibiton of endogenous plasminogen activators by elevated levels of PAI-1 in the circulation decreased the time to occlusive thrombus formation in vivo. This conclusion may be corroborated by experiments conducted in transgenic animals with high circulating PAI-1 levels in which the animals developed thrombotic occlusions in tails and the hindquarters (87). Thus, PAI-1 represents a viable target and until specific inhibitors of this SERPIN are developed it will be an open question as to the importance of inhibiting PAI-1 in preventing thrombotic cardiovascular disease.

Interruption In The Interaction Of α2-AP With Plasmin - Thrombolytic agents share a common final pathway which is the elaboration of plasmin which then degrades fibrin. The normal regulatory modulator of plasmin is α2-AP which modulates plasmin activity in the systemic circulation such that plasminemia does not occur and possibly lead to serious bleeding consequences. However, in some situations, such as thrombotic states, it may be prudent to inhibit α2-AP such that plasmin can work more efficiently to degrade fibrin and thus restore blood flow through previously occluded blood vessels. In an attempt to demonstrate the importance of the plasmin/α2-AP interaction in the systemic circulation, rabbits were treated with an antibody to α2-AP and there was an enhancement of thrombolysis with tPA (88). These results suggest that the combination of a clot-specific inhibitor of α2-AP with a thrombolytic drug at reduced dosage may be an effective thrombolytic strategy.

Conclusion - Thrombosis and resulting myocardial infarction involve the interaction of a number of complex processes involving platelet aggregation, coagulation and fibrinolysis. Accelerated platelet aggregation, coagulation and a low fibrinolytic potential represent targets for new therapeutic approaches in the treatment of thrombosis and myocardial infarction. Thus, careful design of inhibitors of these processes and proper evaluation in animal models of thrombosis may ultimately result in the development of highly specific and effective new therapeutic agents. Well controlled clinical trials will ultimately determine the benefit of these agents and will lead to a better understanding of the etiology of thrombosis and resulting myocardial infarction.

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Chapter 11. Serotonin Modulators and Cardiovascular/Gastrointestinal Diseases

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Introduction - Research carried out in the past decade has led to tremendous advances in our understanding of both the central and peripheral physiological effects of serotonin (5-HT). While the implications of modulation of serotonergic function for the treatment of CNS diseases have been recently reviewed in this series (1,2), the important cardiovascular (CV) and gastrointestinal (GI) effects of serotonin and treatment opportunities afforded by their control have not. Thus , the recent advances in drug discovery efforts toward serotonin modulators for CV and GI disorders form the basis of this chapter. While other reviews (3-10) of serotonin receptors have dealt with aspects of this topic, we will not attempt to duplicate these efforts, but will emphasize newer developments .

It is difficult to explore the area of serotonin pharmacology without encountering the issue of receptor heterogeneity. On this dynamic issue, the Serotonin Club Nomenclature Committee (July 1989) has currently recognized three major classes of serotonin receptors: 5-HT1 receptors, which have a high affinity for serotonin itself; 5-HT2 receptors, which have a low affinity for serotonin; and 5-HT3 receptors,which have moderate affinity (11). The 5-HT1 family is further subdivided into 5-HT1A, 5-HT1B, 5-HT1C, and 5-HT1D subtypes, each of which differ in physiological roles, in distribution and in effector pathways. To date, the 5-HT1A, 5-HT1C, 5-HT1D and 5-HT2 receptors have been cloned and characterized at the molecular level, showing marked differences in protein sequences as well (12-15). Of the high affinity 5-HT1 subtypes of serotonin receptors, the 5-HT1A and 5-HT1D subtypes have been most widely studied with regard to the CV/GI systems. In addition to the "concensus" subtypes, other serotonin binding sites have been proposed and their relevance to CV and GI disorders continues to unfold (16-25).

Although a number of structurally diverse classes of ligands demonstrate high affinity for serotonergic receptors, few selective or highly specific ligands exist for each of the individual receptor subtypes. The search continues for greater specificity, from both the serotonergic receptor subtypes and from other neurotransmitter receptors. The structural diversity permitted by each receptor binding site allows for modification of the chemical and pharmacological properties of the compounds to address such issues as CNS penetration, biovailability and metabolic half-life.

The Importance of Serotonin in Cardiovascular and Gastrointestinal Pathophysiology - Serotonin has a profound and varied effect on most CV tissues. Not only is serotonin found in high concentrations within platelets, but most serotonin receptors have been identified throughout the CV system. With regard to platelets, serotonin can amplify aggregation induced by most aggregating agents by activation of platelet 5-HT2 receptors, an effect associated with increases in phosphoinositide turnover and protein kinase C translocation (26). Serotonin can produce both direct and indirect effects on blood vessels, resulting in vasodilation as well as vasoconstriction (27). Receptors mediating vasodilation include 5-HT1A (28), 5-HT1D (29,30) and possibly 5-HT3 (31) receptors, whereas vasoconstrictor effects of serotonin involve 5-HT2 (7,27) and 5-HT1D (32,33) receptors. Serotonin can produce both bradycardic (5-HT3 receptor mediated) and tachycardic (most all serotonin receptors have been implicated, depending upon species) responses (34). Serotonin can also modify vascular permeability, an effect mediated by activation of 5-HT2 receptors (34). Thus, within the CV system the presence of serotonergic receptors on platelets, blood vessels and the heart implies a vast opportunity for therapeutic intervention in several CV diseases. For example, serotonin has been implicated in the pathogenesis of thrombotic vascular occlusive diseases (35,36). Most recently, data have emerged to suggest that serotonin may be involved in mitogenic responses (37) and in the actions and synthesis or release of growth factors involved in the cellular proliferation associated with atherosclerosis and restenosis (38,39).

With regard to GI systems, the predominant physiological source of serotonin is synthesis within the GI tract. As in the CV system, all major serotonergic receptors have been identified within

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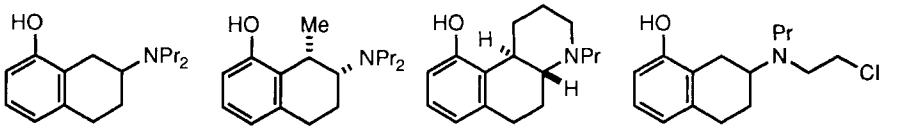
the GI tract, with variations depending upon species and preparation. Most prominent within the GI tract are 5-HT1A (40), 5-HT2 receptors and 5-HT3 (41) receptors in the GI tract. Most recently, 5-HT4 receptors have been identified in intestinal (42) and esophageal (43) tissue. To date, 5-HT3 receptor antagonists have enjoyed clinical success in the treatment of cancer chemotherapy-induced emesis (44). This therapeutic success may pave the way for future utility of serotonergic agonists and antagonists in other GI disorders.

5-HT1A RECEPTOR LIGANDS

Several selective agonists are available at the 5-HT1A receptor, resulting in the rapid expansion of knowledge in this area. To date, most drugs with high affinity at 5-HT1A receptors have been targeted for CNS applications. However, a number of physiologic responses in the GI or CV systems have been recognized as being mediated by 5-HT1A receptors. For example, 5-HT1A receptor agonists can produce bradycardia and hypotension via a central effect (45,46), and activation of 5-HT1A receptors in guinea pig ileum can inhibit neuronally mediated contractile responses (40), raising therapeutic possibilities for peripheral CV or GI effects of 5-HT1A ligands.

Indole-alkylamines - Since 5HT1 receptors have high affinity for serotonin, it is not surprising that several 5HT1A agents share the indole-alkylamine structure of serotonin. Serotonin and its N,N-dimethyl analog bufotenine show little selectivity among the 5-HT1 receptor subtypes, with somewhat higher affinity for 5-HT1A and 5-HT1D sites (47). The closely related 5-carboxamido-tryptamine has 10-fold higher 5-HT1A affinity than does serotonin, but still lacks selectivity versus 5-HT1D receptors. N,N-dipropylation (to afford dipropyl 5-CT) diminishes affinity for the 5-HT1B and 5-HT1C receptors and results in a somewhat 5-HT1A selective ligand (47), suggesting that this tertiary amine substitution is optimal for 5-HT1A receptor affinity and selectivity.

Aminotetralins - The prototypic aminotetralin, 8-OH-DPAT (**1**), is a highly selective 5-HT1A receptor agonist, and as such has been widely utilized as a probe of 5-HT1A receptor mediated activity. However, 8-OH-DPAT lacks stereoselectivity in its interactions with serotonin receptors (48). Structure-activity relationship studies (SAR) based upon this ligand have resulted in the development of stereoselective substituted and conformationally restricted analogs (such as **2** and **3**) which are also potent and selective 5-HT1A agonists and have been used to characterize the recognition requirements of the 5-HT1A binding site (49).

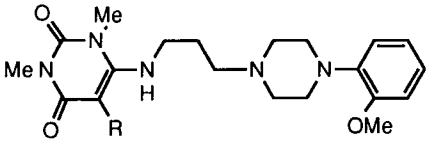


1 **2** **3** **4**

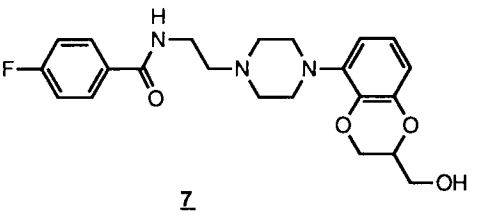
8-OH-DPAT (**1**) can cause hypotension and bradycardia in both conscious and anesthetized rats (45,46). Experiments with pithed animals indicate the involvement of central 5-HT1A receptors (50). The cardiovascular effects could be blocked by the aminotetralin derivative (**4**), a putative 5HT1A antagonist, as well as by the 5-HT1A partial agonist buspirone (50).

Arylpiperazines - Many arylpiperazines show affinity for serotonin receptors. The prototypic arylpiperazines *m*-chlorophenylpiperazine (MCPP) and quipazine lack selectivity and possess affinity for 5-HT1 (all subtypes), 5-HT2 and 5-HT3 receptors (51). Modified arylpiperazines such as buspirone and ipsapirone have been developed as selective 5-HT1A partial agonists (52). Structurally quite different from the aminotetralins, these ligands have also served as the basis for SAR studies (53), as well as computational efforts to map the 5-HT1A binding site (54-56). These drugs have been principally evaluated for their CNS effects (57), although buspirone has been shown to block the cardiovascular effects of 8-OH-DPAT in rats (50). Urapidil (**5**) is another modified arylpiperazine in clinical use an antihypertensive drug for several years, although its mode of hypotensive action is not completely understood (58). Among its multiple activities, urapidil has been shown to be a peripheral α1-adrenoreceptor antagonist (62). Recent work, which correlates the hypotensive actions of urapidil (and its derivatives such as 5-Me urapidil, **6**) with affinity at the 5-HT1A receptor, has suggested that these agents could, in part, exert their effects via a serotonergic mechanism (59-62). In addition, urapidil inhibited both 5-HT and adrenaline-induced human platelet aggregation and the uptake of 5-HT by platelets *in vitro* (63,64). Thus, urapidil is a hybrid drug with multiple activities combined with central 5-HT1A agonist effects (62). Flesinoxan

(7), another modified arylpiperazine which is a potent and selective 5-HT1A agonist, also produced a centrally mediated hypotensive effect in rats (45).



5 R=H
6 R=Me

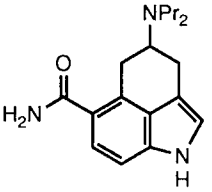


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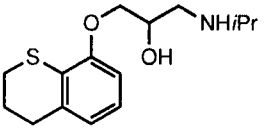
Ergolines and Partial Ergolines - Although primarily investigated as 5-HT2 receptor ligands, several ergolines, including lisuride and lysergic acid diethylamide (LSD), show high affinity for 5-HT1A receptors, although they lack selectivity (47). Some partial ergolines, including LY 178210 (8) and LY 197206 (9), show surprising selectivity as 5-HT1A agonists relative to related ergolines (65). This specificity, however, can be more readily rationalized by recognizing the similarities to the 5-HT1A-selective aminotetralins.



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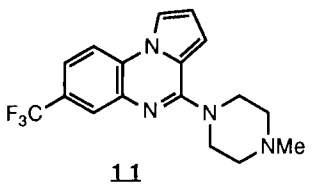
10

Miscellaneous Structures - Pindolol and tertatolol (10), initially studied as beta receptor ligands, have demonstrated 5-HT1A receptor antagonist activity. (-) Pindolol had little effect on CV parameters, but could attenuate the effects of 8-OH-DPAT or those of serotonin (45). Tertatol stereo-selectively ((-) tertatol > racemic > (+) tertatol) induced renal vasodilation in response to 5-HT- or cyclohexyl-adenosine-stimulated vasoconstriction, but not in response to PGF2a-, angiotensin II-, or neuropeptide Y-induced vasoconstriction (66).

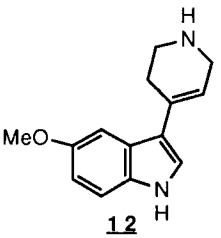
5-HT1B RECEPTOR LIGANDS

While many agents show high affinity for the 5-HT1B receptor, until recently no highly selective 5-HT1B ligands had been identified (51). In addition, the clinical significance of the 5-HT1B subtype remains questionable as the existence of this receptor has yet to be demonstrated in humans (67). Although the 5-HT1B receptor in rodents and the 5-HT1D receptor in humans have been proposed to serve similar functions, little correlation exists between the binding affinities at the 1B and 1D sites (51), nor have 5-HT1B-like vascular receptors been reported in rodents analogous to the 5-HT1D-like sites in certain canine and human blood vessels.

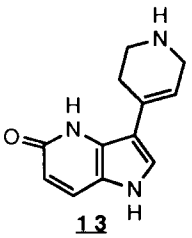
The arylpiperazines MCPP and quipazine show significant affinity but lack selectivity for the 5-HT1B receptor. CGS-12066B (11) has been reported as a 5-HT1B receptor selective



11



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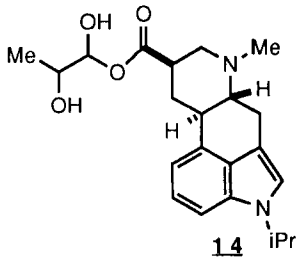
arylpiperazine (68) but recently this selectivity has been questioned (69). The most selective of the 5-HT1B agents yet identified are the tetrahydropyridines, with RU-24969 (12) as the prototypic

ligand (70). Based upon this structure, the rotationally restricted analog CP-93,129 (**13**) is more selective for the 5-HT1B receptor, although somewhat less potent (69).

5-HT1C RECEPTOR LIGANDS

The lack of 5-HT1C selective agonists and antagonists has hindered the investigation of the CV/GI effects mediated by this receptor. However, with available ligands, this receptor has not yet been identified in CV or GI tissue. The 5-HT1C receptors' vascular effects may be involved in initiation of migraine (71). Many of the non-selective 5-HT1 ligands show high affinity for the 5-HT1C receptor. In addition, due to the similarities between the 5-HT1C and 5-HT2 receptors (72), several high affinity ligands for the 5-HT2 receptor also bind potently to the 5-HT1C receptor (47).

Among the indole-alkylamines, 5-methyltryptamine and 1-methyl-5-OH-tryptamine show greater affinity for the 5-HT1C receptor than does serotonin (47). The arylpiperazines MCPP and quipazine have been utilized as 5-HT1C agonists, although as previously described, they are far from selective (51). The "selective" 5-HT2 antagonist mianserin is also an arylpiperazine with high affinity at the 5-HT1C receptors (47). Ergolines such as mesulergine, metergoline, and LY 53857 (**14**), originally characterized as 5-HT2 antagonists, also possess high affinity for the 5-HT1C receptor (47), and LSD is a partial agonist at this site (73). From an SAR perspective, a partial ergoline such as LY 178210 (**9**) does not retain this high affinity for the 5-HT1C receptor (65). The precise contribution of the 5-HT1C receptor binding affinity to the pharmacological effects of these ergolines is not known at this time.

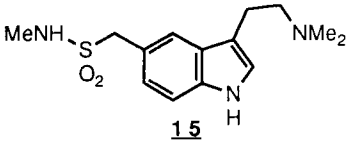


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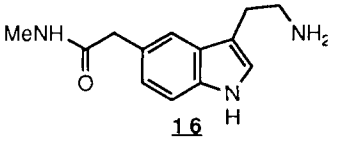
5-HT1D RECEPTOR LIGANDS

The availability of more selective agonists for 5-HT1D (or "5-HT1D-like") receptor has facilitated study of the CV effects mediated by this receptor subtype. 5-HT1D receptor agonists have vasoconstrictor and vasodilator effects in vascular tissues including saphenous vein, basilar artery and coronary artery, and are in clinical study for the treatment of migraine headaches (74).

Indole-alkylamines - Many indole-alkylamines possess high affinity for the 5-HT1D receptors (see 5-HT1A). Stimulating both 5-HT1-like and 5-HT2 receptors, 5-CT induced contractions in human coronary arteries (75). This agent induced tonic contractions in canine terminal ileum and ileocolonic junction, indicating the presence of excitatory 5-HT1-like receptors in intestinal smooth muscle (76). However, because of the lack of selectivity of 5-CT, it is unclear which of its effects are due to 5-HT1D-like receptor activation. A more selective 5-HT1D agonist, sumatriptan (GR43175, **15**), has been developed as an anti-migraine agent (74). Sumatriptan blocked neurogenically mediated plasma protein extravasation in blood vessels of dura matter (77) and caused contractions in human basilar artery (78) and in human saphenous vein (79). A role for selective antagonists of the saphenous vein vasoconstrictor response has been suggested to treat complications of coronary bypass surgery using such veins (79). Sumatriptan, along with the structurally related 5-HT1D agonist AH-25086 (**16**), has been reported to be highly effective in treatment of acute migraine headaches (74). However, the precise role of 5-HT1D-like receptor activation and the locus of sumatriptan's effect in alleviating migraine headache have not been established.



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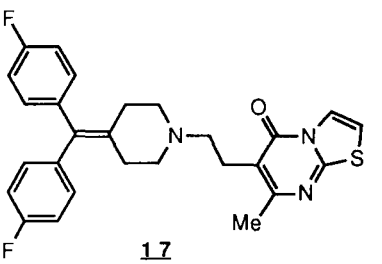
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Miscellaneous Structures - Many ergolines, particularly metergoline and methysergide have high affinity for the 5-HT1D receptor, while other ergolines such as mesulergine and the partial ergoline LY 178210 (**9**) lack significant affinity at this receptor subtype (65). The yohimbine alkaloid isomers yohimbine and rauwolscine , while not selective for the 5-HT1D receptor, are high affinity ligands at the 5-HT1D receptor (47).

5-HT2 RECEPTOR LIGANDS

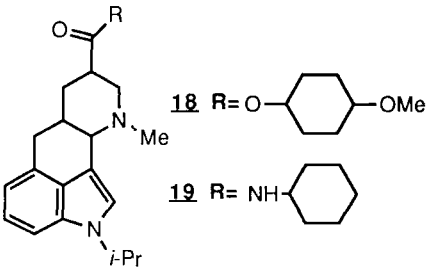
The serotonin-induced pressor response in most arterial systems and serotonin-induced platelet aggregation result from activation of 5-HT2 receptors (7). Thus, 5-HT2 antagonists have been evaluated as potential antihypertensives by virtue of their ability to both retard 5-HT-induced platelet aggregation, and to block the direct contractile effects of platelet-released 5-HT on vascular smooth muscle (80). The important role of platelet 5-HT and 5-HT-induced vasoconstriction has raised the possibility that 5-HT2 receptor antagonists may be effective in clinical thrombosis and other vascular occlusive disorders. This concept has been fueled by the numerous studies of 5-HT2 receptor antagonists in several animal models of thrombosis (35,36). More recently, evidence has emerged linking 5-HT to the ischemic process, again via 5-HT2 receptors; thus, the concept of counteracting the triad "vasoconstriction-thrombosis-edema" via 5-HT2 antagonist therapy has been proposed for stroke management (81).

Modified Butyrophenones - An antagonist at both α-1 adrenoreceptors and 5-HT2 receptors, ketanserin has antihypertensive effects in animals, including humans (82), although considerable controversy remains as to the mechanism(s) by which ketanserin exerts this effect on blood pressure (83,84). Investigators have questioned the role of 5-HT2 receptor blockade, citing the inability of ritanserin (**17**), a more selective 5-HT2 antagonist, to lower blood pressure (85). Both ritanserin and ketanserin similarly antagonized the 5-HT2 mediated pressor response to 5-HT, discounting the possibility that peripheral selectivity of ketanserin and central selectivity of ritanserin might be responsible for the differences in hypotensive effect (86). Ketanserin has recently been shown to attenuate the effects of angiotensin II on rat hemodynamic and vasopressin responses (87), to block the contractile effects of 5-HT on human placental vessels (88) and to inhibit 5-HT-induced contraction of the human mammary artery (89). In addition, ketanserin induced a rise in digital blood flow in patients with Raynaud's phenomenon in a manner not correlated with 5-HT2 receptor blockade of platelet aggregation, consistent with the possibility that α-1 receptor blockade may be responsible for the increased digital flow (90). More recently, 5-HT2 antagonists have been examined for effectiveness in animal models of thrombosis. In this regard ritanserin also inhibited acute platelet thrombosis in stenosed coronary arteries of dogs with no effect on heart rate or blood pressure (35).



17

Ergolines - Ergolines such as methysergide, metergoline and mesulergine are non-selective blockers of 5-HT2 receptors (47). Ergoline 5-HT2 receptor antagonists such as LY 53857 (**14**), sergolexole (LY 281067, **18**) and LY 237733 (**19**), which lack affinity for adrenergic, histaminergic and dopaminergic receptors, also potently inhibit 5-HT stimulated human platelet aggregation (91). Like ritanserin, these 5-HT2 receptor ergolines that lack α-1 receptor blockade do not lower blood pressure in animals (92,93). In addition, LY 53857, which can enter the CNS, blocked the increases in plasma renin activity, arterial pressure increases and decreases in renal blood flow to both i.c.v and i.v. administered quipazine, a direct 5-HT agonist, while the peripherally selective 5-HT2 antagonist xylamidine blocked only the renin and renal blood flow (rbf) effects, and failed to block the arterial pressure increases (94). Thus, these effects of quipazine were due to 5-HT2 (or 5-HT1C) receptor activation, and the renin and rbf effects were peripherally mediated. The individual diastereomers of LY 53857 (meso, RR and SS configurations of the ester side chains) each display similar potency and selectivity as 5-HT2 antagonists (47,95). Furthermore, like ritanserin (51), LY 53857 was effective in alleviating vascular occlusive effects in animal models of thrombosis (36). For related ergolines, the indole N-isopropyl substituent is optimal for vascular 5-HT2 receptor affinity (96). Although some ergolines may possess selectivity for 5-HT2 receptors, most ergolines also have high 5-HT1C receptor affinity.

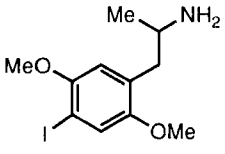


18 R= O—⬡—OMe

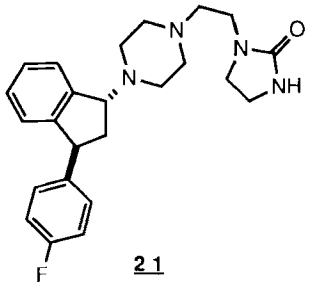
19 R= NH—⬡

Miscellaneous Structures - The arylpiperazine quipazine produces transient hypotension and bradycardia followed by sustained hypertension and variable effects on heart rate in anesthetized

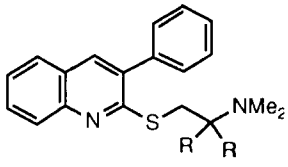
rats via both 5-HT2 and 5-HT3 receptors (97). Somewhat selective 5-HT2 partial agonists (such as DOI, **20**), although still possessing relatively high affinity for 5-HT1C receptors (47), have been discovered based upon dimethoxyphenylaminopropane and have been utilized to study the recognition requirements of the 5-HT2 receptor (98). Although studied primarily for its behavioral effects, **20**, via 5-HT2 receptors in vascular smooth muscle and in brain, increased arterial pressure with reflex bradycardia (99). Decreases in renal blood flow and increases in plasma renin activity also occurred and were blocked by LY53857 (**14**), a 5-HT2 antagonist which can enter the brain, or by the peripheral 5-HT2 antagonist xylamidine. A recently developed indanylpiperazine 5-HT2 receptor antagonist, irindalone (**21**) had marked hypotensive effects in rats (100). The preclinical pharmacology of irindalone is quite similar to that of ketanserin, with affinity for α-adrenergic receptors as well as 5-HT2 receptors. The effects of irindalone show dramatic stereoselectivity, as its enantiomer (Lu 21-099) failed to block 5-HT2 receptors (101).



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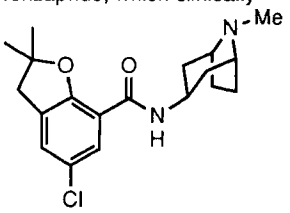
22 R=H
23 R=Me

The novel quinoline 5-HT2 receptor antagonist ICI 169,369 (**22**) inhibited 5-HT-induced vascular contractions at both ketanserin-sensitive and -insensitive sites (102). In addition, **22** antagonized the 5-HT-induced bronchoconstriction in the guinea pig and 5-HT-induced pressor responses in anesthetized dog (103). Clinical evaluation of this agent for acute and prophylactic treatment of migraine headaches showed only modest efficacy (104), possibly related to first pass metabolism in man (105). ICI 170,809 (**23**) is a more recent analog which may lack this metabolic lability (106). Naftidrofuryl, a 5-HT2 antagonist which inhibits 5-HT-induced vasoconstrictor and platelet aggregation (107-109), has clinical efficacy in both acute and subacute stroke patients (110,111). Whether the efficacy of naftidrofuryl resulted from 5-HT2 receptor antagonism or from other effects of this compound is not clear (112).

5-HT3 RECEPTOR LIGANDS

Interest in the classical "M" receptor of Gaddum and Picarelli has never been higher than at present. Since the report of MDL 72222, the first selective 5-HT3 antagonist (113), the field has exploded with agents exhibiting 5-HT3 antagonist properties. Although 5-HT3 receptor antagonists were initially developed to block the acute pain of migraine (114), the principle clinical application for 5-HT3 antagonists is suppression of chemotherapy-induced emesis, a topic recently reviewed (115). Recent findings, however, indicate that many 5-HT3 receptor antagonists also have high affinity for the 5-HT4 receptor, questioning the relative importance of the 5-HT3 (versus 5-HT4) receptor interactions to the observed pharmacology.

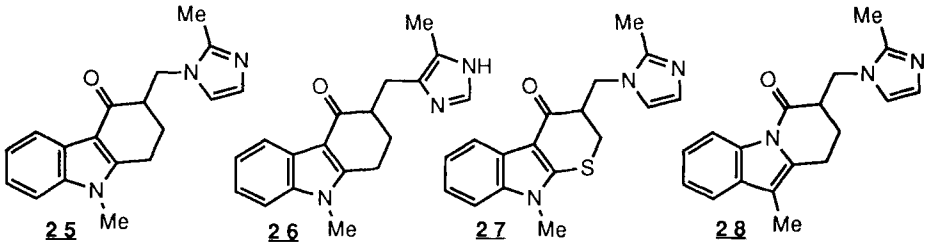
Tropanes and "Tropane-like" Structures - Based upon the structure of cocaine, a weak non-selective 5-HT3 antagonist, selective high affinity 5-HT3 antagonists have been synthesized, the first among them being the benzoic acid derivative MDL 72222. SAR studies revealed considerable latitude among the benzoates and benzamides of "tropane-like" tertiary amines for 5-HT3 receptor affinity. Among the benzoic acid-derived agents are zacopride and renzapride, which clinically suppress chemotherapy-induced emesis (115). In conscious ferrets, zacopride produced a number of gastrointestinal side effects, including emesis and defecation (116), possibly via interaction with 5HT4 receptors (117). ICS 205-930, in which the aryl moiety is an indole-3-carboxylate, is also a potent 5-HT3 antagonist which has clinical antiemetic efficacy (118). The effects of ICS 205-930 on gastric emptying and antral motor activity suggest only slight effects on gastric motor activity in healthy male volunteers (119), although this compound has



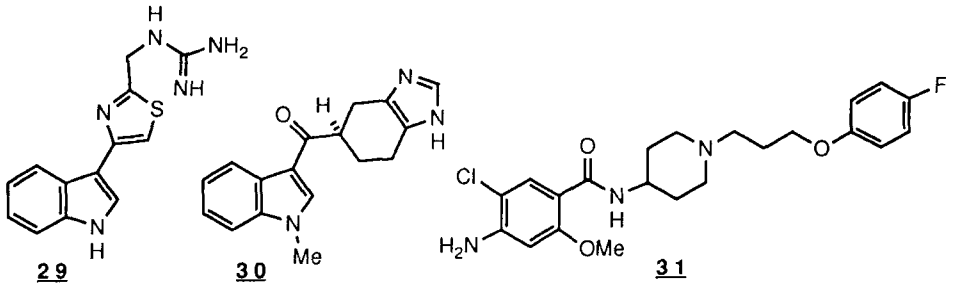
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relatively modest affinity at 5-HT4 receptors. Intestinal secretion activated by cholera toxin was partially reduced by ICS 205-930, but when combined with the 5-HT2 receptor antagonist ketanserin, total block of the effect of cholera toxin occurred, suggesting involvement of both 5-HT2 and 5-HT3 (or 5-HT4) receptors in gastrointestinal secretory responses (120). The closely related indazole granisetron is also a clinically effective antiemetic (121-123). Some 5-HT3 antagonists can differentiate between the antiemetic and gastric prokinetic effects. For example, zatosetron (**24**) is a potent 5-HT3 receptor antagonist effective in inhibiting cisplatin induced emesis in dogs, which lacks effect on gastric emptying in rats (124).

Carbazolones and Related Structures - The prototypic carbazolone 5-HT3 antagonist ondansetron (GR 38032, **25**) , is also clinically effective against chemotherapy-induced emesis (125,126). In human clinical trials, ondansetron was no different from placebo on gastric emptying or on mouth-to-cecum transit times. However, the drug did significantly increase whole-gut transit time (127,128). Additional SAR studies of ondansetron have resulted in identification of several related high affinity 5-HT3 receptor antagonists including GR 67330 (**26**) and the recently disclosed (129-131) compounds (**27**) and (**28**).



Miscellaneous Structures - Although no specific 5-HT3 receptor agonists have been identified, 2-methyl-5-HT and phenylbiguanide display relatively high potency and some selectivity for the 5-HT3 receptor (132). Utilizing the structures of these agonists and the antagonists described previously, several groups have proposed pharmacophore models for the 5-HT3 receptor binding site (133-135). Based upon the models, novel agents with little resemblance to the original "tropane" cocaine structure , such as 3-[2-(guanidinylmethyl)-4-thiazolyl]indole (**29**), block the Bezold-Jarisch reflex in rats (136). YM 060 (**30**) is the newest of the highly selective high affinity 5-HT3 antagonists which conform to the proposed pharmacophore models (137).



5-HT4 RECEPTOR LIGANDS

Although some of the earlier 5-HT3 antagonists possessed both antiemetic and gastric prokinetic effects, recent data suggest that the prokinetic effects of these agents are not mediated via 5-HT3 receptors (117). A receptor possibly associated with this enhanced gastric motility has been identified in the guinea pig ileum and has been designated 5-HT4 (42). This 5-HT4 receptor has been associated with 5-HT-induced tachycardia in pig (138), has been recently reported to mediate positive inotropic effects in human myocardium (139) and has been characterized in mouse embryo colliculi neurons (140). The principle agents with high affinity at the 5-HT4 receptor are the benzamides such as cisapride (**31**), renzapride and zacopride, compounds also possessing affinity at 5-HT3 receptors, and which behave as partial agonists at the 5-HT4 receptor (140). ICS 205-930 is a weak antagonist (140). While no selective full agonists for the 5-HT4 receptor have been identified, 5-methoxytryptamine and α-methyl-5-HT mimic the 5-HT-induced tachycardia mediated

by the pig cardiac 5-HT4 receptor (138). The precise role of 5-HT4 receptor agents in CV and GI disease awaits the development of more selective agents that do not bind to 5-HT3 receptors.

Conclusions - Rapid progress continues in the arena of serotonin-based therapeutics for CV and GI disorders. With more selective ligands for each of the individual serotonin receptor subtypes, the complex pharmacology of serotonergic systems and their role in CV and GI physiology will continue to unfold and should offer multiple drug development opportunities (141,142).

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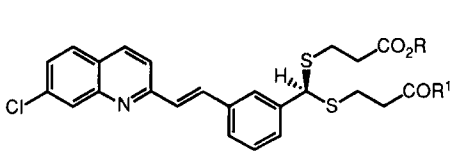
Chapter 12. Pulmonary and Anti-Allergy Agents

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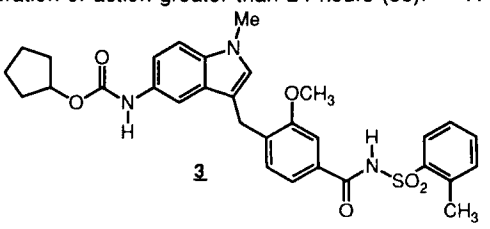
Introduction - Pharmacotherapeutic agents targeted for bronchial asthma continue to dominate drug discovery efforts in the pulmonary area. Deaths and morbidity from asthma have risen world-wide (1). Asthma has been further characterized as a disease associated with chronic inflammation (2-4), which may underly the prevalent bronchial hyperresponsiveness in asthmatics (5). The role of mast cells (6,7) and eosinophils (8-10) in airway inflammation and hyperresponsiveness have been elucidated further. Antagonism of intercellular adhesion molecule 1 (ICAM-1) has been suggested as a novel therapeutic approach to reduce airway hyperresponsiveness (11). A soluble form of ICAM-1, which is normally membrane bound, potently inhibits human rhinovirus infection (12). Neural control of airway caliber and indirect effects of neurotransmitters contributing to airway inflammation continue to be investigated (13,14). Important advances in understanding the role of peptides (15), adrenergic receptors (16) and muscarinic receptors (17) and autoreceptors (18) in the airways have been made. Overexpression of a human chimeric α subunit of the high affinity IgE receptor (FcERI) has presented the opportunity for identifying specific antagonists of the IgE/FcERI binding interaction, which may prove to be effective in the treatment of allergic diseases (19).

REGULATORS OF LIPID MEDIATORS

Leukotriene Antagonists - Preliminary evidence from clinical studies continues to support a significant role for leukotrienes (LT's) in asthma (20). In a placebo-controlled trial, the potent LTD_4 receptor antagonist MK-571 attenuated exercise-induced bronchoconstriction in subjects with stable asthma (21). Preparation and evaluation of the two enantiomers of MK-571 (S-(+)-isomer **1**; R-(-)-isomer **2**) revealed comparable binding to isolated human lung membranes (3). The (+)-isomer (pK_B = 9.0) was slightly more potent than the (-)-isomer (pK_B = 8.7) as an antagonist of LTD_4 contractions in guinea pig (GP) trachea (22). Comparison of the blood levels of both isomers revealed that the (-)-isomer gave higher levels at all time points and was cleared less rapidly than the (+)-isomer (23). The genesis of the bicyclic ring nucleus and refinement of the acidic region in a series of indazole and indole LT antagonists ultimately led to the discovery of potent LTD_4 antagonist ICI-204,219 (**3**), the medicinal chemistry of which has been recently reported (24-26). A striking generality of the central bicyclic ring as a template in a follow-up series of compounds related to **3** was observed (27). The preclinical and early clinical pharmacology of **3** have been reported (28-30). Synthesis and pharmacology of the LT antagonist SKF-104353 (**4**) have been reviewed (31). The leading compound from a series of (2-quinolylmethoxy)phenyl-containing orally active LT receptor antagonists, RG-7152 (**5**), gave a K_i of 42 nM in an ^3H-LTD_4 binding assay in GP lung membranes (32). Introduction of a phenyl ring spacer between the lipophilic (2-quinolinylmethoxy)phenyl moeity and the acidic component, exemplified by RG-12525 (**6**), improved receptor binding (33). Further evolution of the series led to a potent benzopyran derivative, RG-12553 (**7**), which exhibited a K_i of 0.1 nM (34). Another new LT antagonist containing the benzopyran group, CGP-45715 (**8**), appears to have potency comparable to that of **3**, but with a duration of action greater than 24 hours (35). A



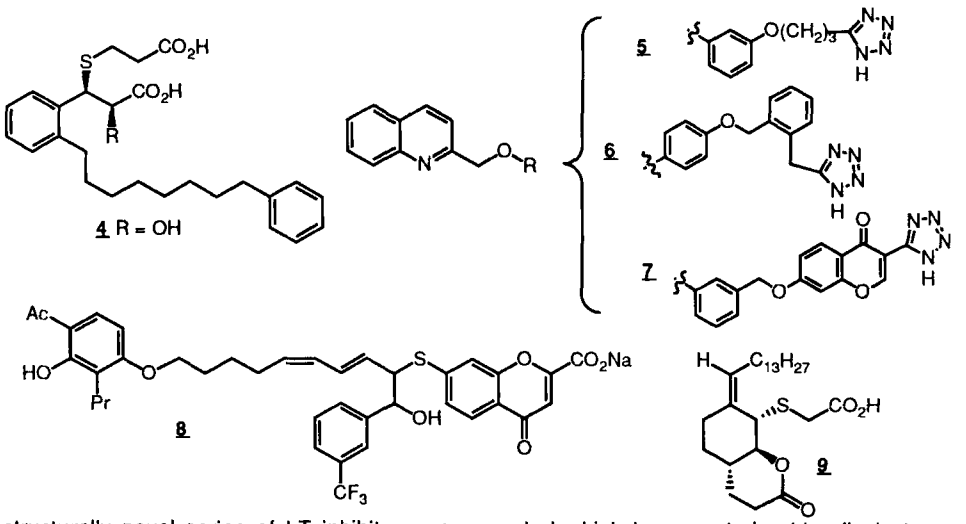
1 R = NMe_2, R^1 = OH
2 R = OH, R^1 = NMe_2



3

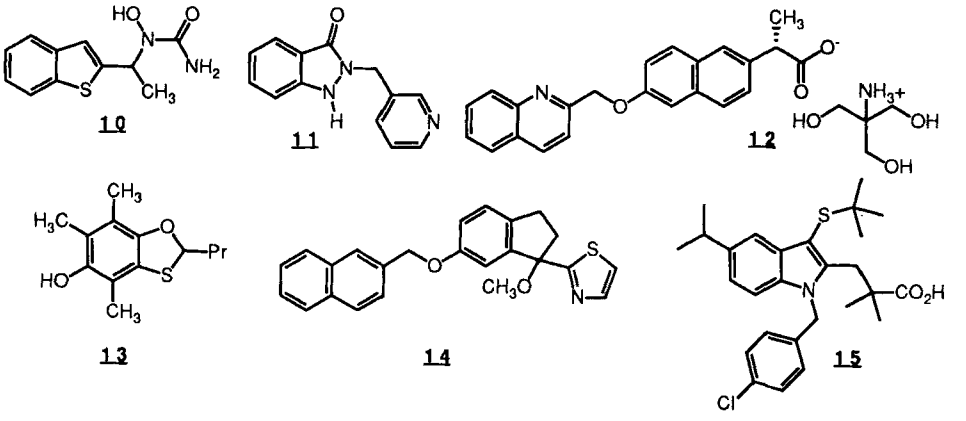
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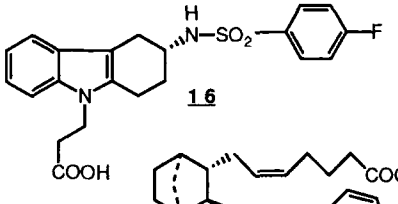
structurally novel series of LT inhibitors was reported which incorporated a bicyclic lactone. MDL-43,291 (9) is a competitive antagonist of LTD_4 (pA_2 = 6.7) and LTE_4 (pA_2 = 6.7) and an apparent non-competive inhibitor of LTC_4 ("pA_2" = 6.8), all in the GP ileum (36). Cysteinyl leukotrienes have been reported to proliferate epithelial cell growth in concentrations as low as 10 fM, possibly representing a new important physiological role for the LT's (37).

5-Lipoxygenase Inhibitors - Results from 5-lipoxygenase (5-LO) inhibition in man have been reported. In a double-blind, randomized study of the 5-LO inhibitor zileuton (A-64077, 10) in asthmatic males, airway reactivity to hyperventilation of cold, dry air was reduced following oral administration of the drug (38). This indicates that attenuation of the asthmatic response to cold, dry air may be accomplished through selective inhibition of 5-LO. Allergen-induced nasal congestion was significantly decreased by 10 in human volunteers, suggesting the potential therapeutic importance of 5-LO inhibition in allergic rhinitis (39). Evaluation of the QSAR for a number of related hydroxamic acids revealed a close relationship between hydrophilicity and 5-LO inhibitory potencies (40). A series of 2-substituted indazolones were identified as potent and selective inhibitors of 5-LO (41). The 2-pyridylmethyl analog ICI-207968 (11) inhibited LTB_4 release from calcium-ionophore stimulated blood from rats after oral dosing with no inhibition of PGE_2 synthesis at oral doses up to 300 mg/kg. The pharmacology and chemistry of the mixed 5-LO inhibitor/leukotriene antagonist WY-50295 (12) have been described (42-44). In ovalbumin-sensitized guinea pigs, 12 tromethamine elicited a dose-dependent inhibition of bronchoconstriction when given orally (45). Orally dosed 12 antagonized LTD_4-induced bronchoconstriction in guinea pigs (46). 12 appears to be 100% bioavailable with an

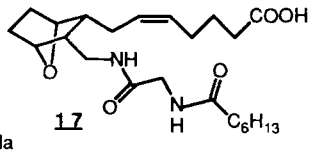


extended duration of activity (43). Compound **13**, from a series of 1,3-benzoxathioles, was both an inhibitor of 5-LO and an antagonist of SRS-A formation or release from antigen provocation in sensitized GP lung slices; **13** has been selected for further development for asthma (47). ICI-216,800 (**14**) exemplifies the first chemical series to show a stereospecific interaction in the inhibition of 5-LO (48). The synthesis of LTB_4 in human blood was inhibited by (+)-**14** with an IC_{50} of 0.54 μM compared to >40 μM for the (-)-isomer. Selectivity for 5-LO inhibition was determined by inhibition of the LTC_4 synthesis in plasma-free peritoneal macrophage cultures with no inhibition of PGE_2 synthesis. MK-886 (L663,536, **15**), a leukotriene biosynthesis inhibitor, was examined in atopic subjects with documented early (EAR) and late (LAR) asthmatic reactions, and in the subsequent increase in airway hyperresponsiveness to histamine (49). Given orally, **15** inhibited the antigen-induced EAR and delayed the LAR, but did not afford protection against the increase in airway hyperresponsiveness.

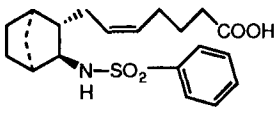
Thromboxane-related Studies - Preliminary clinical studies of thromboxane A_2 receptor antagonists (TXRAs) and thromboxane synthetase inhibitors (TXSIs) have been reviewed and suggest that TXRAs offer greater clinical potential than TXSIs (50). The synthesis and absolute configuration of a new TXRA, BAY-U-3405 (**16**), was described (51); **16** is a selective *in vivo* antagonist of TXA2-induced bronchoconstriction and inhibits U46619-induced contractions in a variety of tissues (52,53). Bicyclic prostaglandin analogue SQ-30,741 (**17**) is a potent and selective TXRA which is free of overt toxicity (54,55). Molecular modelling analysis of the putative active conformations of thromboxane A_2 and U46619 provided a working hypothesis of the molecular structural requirements for a potent TXRA (56). These conformational requirements were satisfied by S-145 (**18**), a compound identified as a very potent TXRA. Azulene derivative **19** was a potent antagonist of U-44619-induced constriction in rat aorta and was orally effective against U44619-induced sudden death in mice (57). Potent and selective TXSI OKY-046 was orally effective against antigen-induced bronchoconstriction and airway hyperresponsiveness in guinea pigs (58,59). Moreover aerosol delivery of OKY-046 to human asthmatics reduced bronchial responsiveness to acetylcholine (60). Compound Y-20811 has been characterized as a TXSI and dose-dependently inhibited bronchoconstriction induced in guinea pigs by arachadonic acid, leukotriene D4 and platelet-activating factor (61). The pharmacokinetics and pharmacodynamics of DP-1904 in man after repeated oral doses indicate that the compound is a potent and long-acting TXSI (62,63).



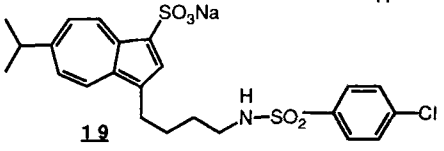
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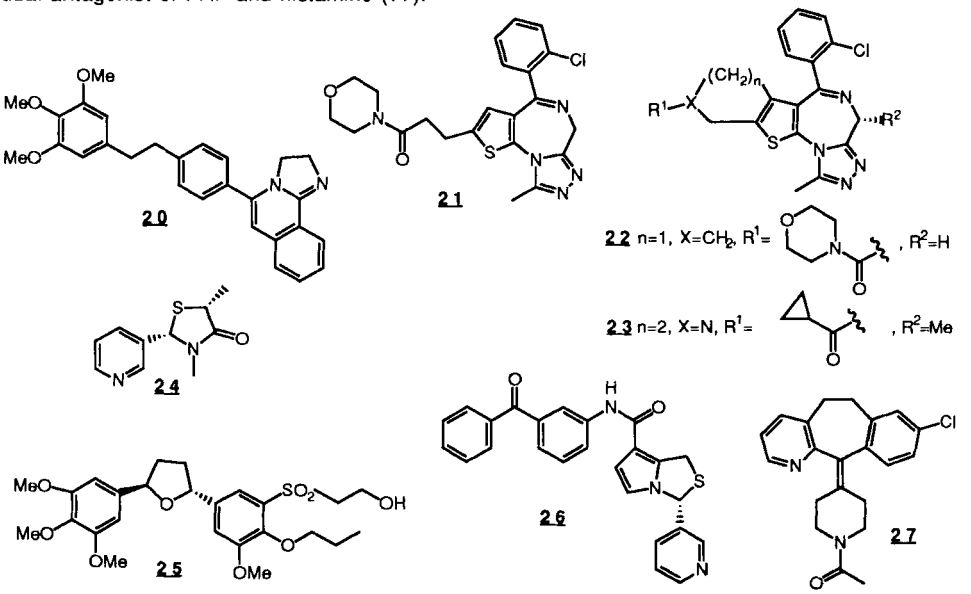
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19

PAF Antagonists - The effects of platelet-activating factor (PAF) on cells and tissues have been reviewed (64). PAF has been implicated as an important mediator in a variety of allergic and inflammatory reactions, including asthma. The PAF antagonist SDZ 64-412 (**20**) inhibited the airway hyperresponsiveness induced by antigenic challenge of previously sensitized guinea pigs (65). However, a study attempting to correlate asthmatic activity with PAF levels in bronchoalveolar lavage fluid from human asthmatics tentatively concluded that PAF was not the only factor responsible for maintaining bronchial hyperresponsiveness (66). Encouraging clinical results came from a double-blind, placebo-controlled trial in which apafant (WEB-2086, **21**) protected healthy volunteers from PAF-induced bronchoconstriction and cardiovascular changes (67). Hetrazepine analogues WEB-2170 (**22**) and E-6123 (**23**) have emerged as PAF antagonists with better potency and pharmacokinetics than **21** in animal models (68-72). SM-10661 (**24**) is highly water soluble, inhibits alkyl-PAF-induced aggregation of human platelets, and showed rapid absorption and good duration of action following oral dosing to rats and

rabbits (73). MK-287 (L-680573, **25**) inhibited PAF-induced platelet aggregation, PAF-induced lethality in mice and PAF-induced bronchoconstriction in guinea pigs (74). Potent PAF antagonist RP-59227 (**26**) was synthesized stereospecifically (75), and was utilized to investigate the effects of plasma protein content and platelet number on the potency of PAF as a platelet aggregatory agent (76). SCH-37370 (**27**) is the first compound to be identified as a dual antagonist of PAF and histamine (77).

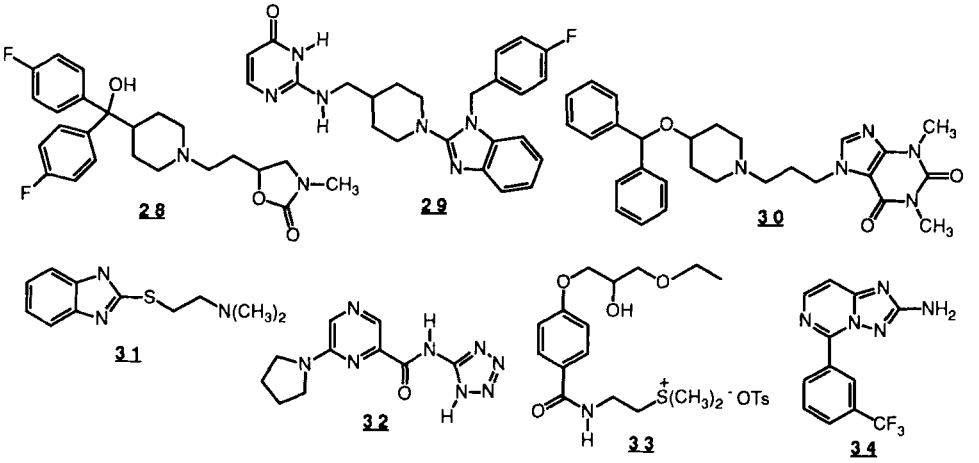


ANTIALLERGICS

Non-Sedating Antihistamines - Nasal provocation studies in man have indicated that histamine levels rise in a biphasic manner. During the early phase, the rise in histamine appears to be associated with activation of mast cells, while the late phase rise in histamine is concomitant with an increase in the number of basophils (78). The potent, long-acting non-sedating antihistamine AHR 14310C (**28**) inhibited antigen-induced mucus formation in rats at low oral doses (79). Such antagonism of mucus formation is not inhibited by classical antihistamines or by the newer non-sedating antihistamines, thus representing a significant potential improvement upon existing therapy. Another non-sedating antihistamine, SL 850324 (**29**), inhibited the ovalbumin-induced passive cutaneous anaphylactic reaction in rats and protected rats from compound 48/80-induced lethal shock (80). The H_1 antagonist Wy-49,051 (**30**) competitively and very potently antagonized histamine-induced contractions in GP ileum (pA_2 = 11.1) and appeared to have less potential for producing sedation compared to ebastine or terfenadine (81). DF-10967 (**31**) showed potent inhibition of ^{3}H-mepyramine binding and protected against compound 48/80-induced lethality in rats (82). Poor penetration into the CNS and a lack of interaction with other receptors may account for the lack of sedation observed with **31**.

Mediator Release Inhibitors - The mechanism of action of the pyrazinecarboxamide derivative HSR-6071 (**32**) was reported to involve the suppression of release of histamine and SRS-A in addition to some inhibitory effects on cAMP phosphodiesterase activity (83). Activity in the PCA assay in rats indicated **32** to be orally active (84). Suplatast tosylate (IPD-1151T, **33**) was demonstrated to suppress human IgE synthesis through inhibition of the release of IL-4 and IL-6 from antigen-activated T cells, and appeared to markedly improve clinical symptoms in allergic subjects in conjunction with a decrease in serum IgE levels (85). Using a human basophil histamine release assay, a series of triazolo[1,5-c]pyrimidines were found to be active as mediator release inhibitors (86). Lead compound **34** gave an IC_{50} of 2.2 μM in the basophil assay and was active in the mouse PCA assay. Immunization of rabbits with a human decapeptide

sequence from the CH4 domain of human IgE, after conjugation to keyhole limpet hemocyanin, was shown to inhibit histamine release from rat mast cells both in vivo and in vitro (87).



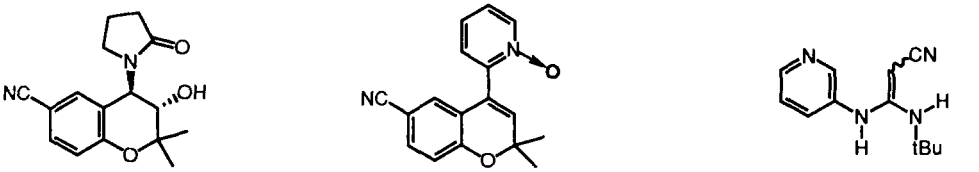
28 29 30

31 32 33 34

OTHER TOPICS

β_2-agonists - Inhaled β_2-agonists are effective bronchodilators which have achieved wide therapeutic use against asthma. The clinical pharmacokinetics of ß-agonists have been reviewed (88). The early β_2-agonists, such as salbutamol, act rapidly but display short duration. Worldwide introduction of the long-acting β_2-agonists salmeterol and formoterol represents a significant development (89,90). While these new agents display long duration of action, they are slow to achieve peak bronchodilation (91,92). They are effective against nocturnal asthma, and in contrast to early β_2-agonists the new drugs appear to block the late phase response to allergen challenge (93,94). The arrival of these new agents has exacerbated the controversy over the clinical role of β_2-agonists in asthma (95-97).

Potassium Channel Openers - Potassium channel openers (PCO's), reviewed in detail in chapter 8, continue to be of interest in airway dysfunction (98,99). A study involving the oral dosing of cromakalim to asthmatics indicated a significant decrease in the early morning fall in FEV_1 suggesting therapeutic potential in nocturnal asthma (100). The more potent (3S,4R)-isomer, lemakalim (**35**), is being developed clinically (101). The novel PCO Ro 31-6930 (**36**) is about 10 times more potent than cromakalim in blocking histamine-induced bronchoconstriction in guinea pigs (102). A comparative study of four PCO's in relaxing GP trachea and in blocking 5-HT-induced bronchospasm indicated the tracheal relaxant potency in vitro correlated closely with their bronchodilator activity (103). The most potent compound, P1060 (**37**), was 3.5-4 times more potent than cromakalim and about 20 times more potent than pinacidil.



35 36 37

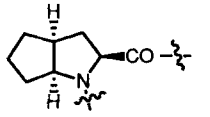
Bradykinin Antagonists - Bradykinin (BK) receptor antagonists have recently been reviewed (104-106). A BK_3 receptor, believed to be involved in mediating bronchoconstriction, has been identified in guinea pig (GP) lung membranes (107,108). Two new, highly potent BK_2/BK_3 receptor antagonists were recently reported. In GP trachea, HOE-140 (**38**) antagonized BK with an IC_{50} of <100 nM (109). A pA_2 of 8.82 was measured in the GP ileum against BK-induced contractions (110). A series of peptide analogs related to HOE-140 were prepared to

investigate the hypothesis that peptide BK-receptor antagonists must form a β-turn at their C-terminus to elicit high receptor affinity (111,112). The most potent derivative, **39**, bound to GP ileal membranes with a K_i of 0.176 nM and gave a measured pA_2 of 9.4 against BK-induced synthesis of prostaglandins in SV-T2 fibroblasts.

(D)Arg-Arg-Pro-Hyp-Gly-Thi-Ser-(D)-Tic-Oic-Arg

38

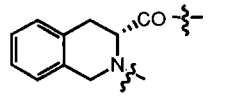
Oic =



(D)Arg-Arg-Pro-Hyp-Gly-Thi-Ser-(D)-Tic-Tic-Arg

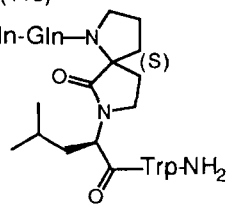
39

Tic =



Tachykinins - The physiological effects of the tachykinins, in particular substance P (SP) and neurokinin A (NKA), mirror the pathophysiology underlying asthma and may contribute to bronchial hyperreactivity (113). Recent molecular cloning of the rat SP receptor (114), and the NK_2 receptor from human tracheal and gastric tissues (115) have been reported, establishing the tachykinin receptors in the growing family of structurally related G-protein coupled receptors. SP analog **40**, which incorporated a conformationally restricting spiro-fused lactam, gave a pK_B of 7.70 against the NK_1 receptor agonist SP methyl ester in GP ileum (116). NKA analog MDL-28,564 (**41**) has been exploited to distinguish between apparent NK_2 receptor subtypes (117). In GP tracheal tissues, **41** stimulated phosphatidyl inositol (PI) turnover and induced contractions similar to the full agonist NKA. However, in hamster urinary bladder tissues (HUB), **41** was a competitive antagonist of NKA-induced PI turnover and did not produce a sustained contraction in HUB. This suggested two types of NK_2 receptors (117). In another series of peptide-based antagonists of NKA, NK_2 receptor heterogeneity was further implicated with peptide **42** being more potent in blocking NKA-induced contractions in hamster trachea (pA_2 = 7.67) compared to rabbit pulmonary artery (pA_2 = 5.42), both purported NK_2-receptor rich tissues (118)

Arg-Pro-Lys-Pro-Gln-Gln-



-Trp-NH_2

40

Asp-Ser-Phe-Val-Gly-Leu-ψ(CH_2NH)-Leu-NH_2

41

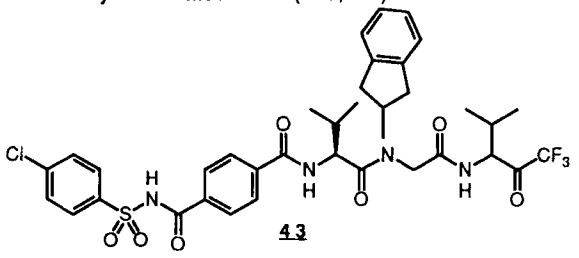
Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2

42

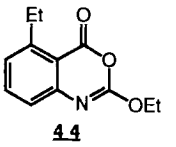
Cystic Fibrosis - Major advances were made in the area of cystic fibrosis (CF), a disease attributable to the presence of cells bearing a defective gene. Two independent groups paved the way for gene therapy by demonstrating that in vitro insertion of the normal gene into human cystic fibrotic endothelial cells corrected the deficit in chloride ion channel opening which is characteristic of the disease (119-121). The defective gene encodes a membrane-associated glycoprotein called cystic fibrosis transmembrane conductance regulator (CFTR) which affects functioning of the ion channel. A recent study demonstrated that mutant forms of CFTR remain incompletely processed and are degraded within cells; lack of mature CFTR at the appropriate membrane location is suggested as the molecular basis for the disease (122). A suggestion has been made that leukotriene LTC_4 and prostaglandin D_2 might be the substrates for CFTR, and that these molecules in turn are the actual regulators for the epithelial chloride channel (123). Clinical trials with aerosolized amiloride, a sodium-channel blocker, demonstrated enhanced mucus clearance in CF patients (124,125). Catalytic amounts of recombinant human DNase were shown to effectively reduce the viscosity of purulent CF sputum ex vivo , thus establishing the basis for another clinical approach (126).

Emphysema - The long-standing "protease-antiprotease imbalance" theory for the development of emphysema has gained further support: the bronchoalveolar lavage fluid from emphysematics was examined, and human neutrophil elastase (HNE) burden correlated directly and antielastase capacity correlated inversely with emphysema (127). Augmentation therapy gained support by the finding that α_1-antitrypsin (AAT) ameliorates HNE-induced emphysema in hamsters (128). The potential therapeutic value was underscored by studies which found increased elastase

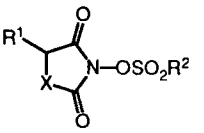
inhibitory capacity (EIC) in the epithelial lining fluid (ELF) of human subjects who had received either aerosol or intravenous (i.v.) doses of AAT (129-131). Recombinant secretory leukoprotease inhibitor (rSLPI) was effective at regulating HNE proteolysis both in vitro and in vivo (132,133). Aerosolized rSLPI was delivered to sheep and elevated EIC in the ELF was observed (134); the pharmacokinetics of rSLPI administered by i.v. injection to humans were explored (135). A peptidyl boronic acid inhibitor of HNE was found to exacerbate HNE-induced emphysema in hamsters, thus raising a caveat for the therapeutic use of these and other synthetic inhibitors in humans (136). The peptidyl α-keto ester Ac-Pro-Ala-Pro-Ala-CO_2Me was more potent as an in vitro inhibitor of HNE than the corresponding trifluoromethylketone (TFMK) Ac-Pro-Ala-Pro-Ala-CF_3, and the compound Nα-(Ad-SO_2)-Nε-(MeO-Suc)Lys-Pro-Val-CF_3 was reported to have a K_i of 0.58 nM versus HNE (137). BIRA-0260 XX (**43**), a TFMK containing an N-substituted glycine residue at the P_2-position, had long duration of action when dosed intratracheally to hamsters prior to HNE challenge (138,139). Extensive studies on cephalosporin analogues have expanded the available data on this series of time-dependent HNE inhibitors (140-142); recent work on monocyclic ß-lactams has demonstrated that these variants are also effective inactivators of HNE (143). 4H-3,1-Benzoxazin-4-ones are alternate substrate inhibitors of HNE, and 2-ethoxy-5-ethylbenzoxazinone (**44**) emerged from this series with K_i = 42 pM (144). Hydantoin derivatives (**45**) and N-hydroxysuccinimide derivatives (**46**) irreversibly inactivated HNE (145,146).



43



44



45 X=NH

46 X=CH_2

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SECTION III. CHEMOTHERAPEUTIC AGENTS

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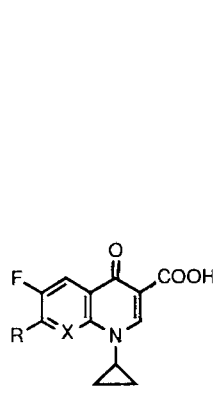
Chapter 13. Antibacterial Agents

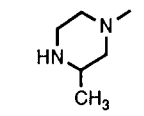
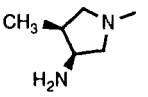
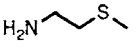
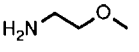
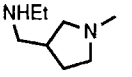
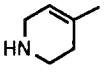
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Pfizer Inc., Central Research Division, Groton, CT 06340

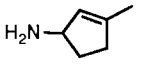
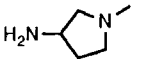
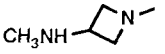
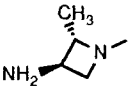
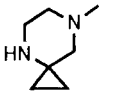
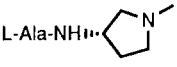
Introduction - Antibacterial research this year has focused on discovery of agents which exhibit improved activity, particularly against resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), and decreased incidence of side effects. The mechanisms of resistance and transport for various classes of antibacterial agents are under vigorous investigation.

Quinolones - Reviews appeared this year covering quinolone agents (1), their human pharmacokinetics and metabolism (2), and clinical utility (3-5). Use of these drugs in children is receiving cautious support, given an appropriate risk/benefit ratio (6,7). Effects of quinolones on bacterial cells (8), central nervous system side effects (9) and structure-activity relationships (10-12) have been reviewed. The development of new compounds has been characterized by an emphasis on structural novelty, improvement of potency versus anaerobic and gram-positive pathogens, incorporation of activity against quinolone-resistant strains, and early assessment of the potential for adverse drug reactions.

Use of N-1 cyclopropyl and C-8 heteroatom substituents continued to provide potent antibacterial agents. AM-1155 (1) and AM-1174 (2), new quinolones bearing an 8-methoxy group, outperformed ciprofloxacin against anaerobes and a range of gram-positive bacteria, with MRSA potency 16- to 62-fold greater than ciprofloxacin. AM-1174 exhibited activity >128-fold that of ciprofloxacin against ciprofloxacin-resistant strains of *S. aureus* (13,14); AM-1155 was comparable to ciprofloxacin in a *Pseudomonas* infection in mice, and was well absorbed orally in a variety of



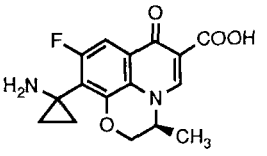
	R	X
1		C-OCH$_3$
2		C-OCH$_3$
3		C-Cl
4		C-Cl
5		C-CF$_3$
6		C-H

	R	X
7		N
8		N
14		C-F
15		N
16		C-F
19		N

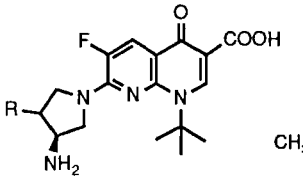
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species (15). In an interesting departure from standard SAR, the 8-chloro derivatives **3** and **4** were found to exhibit *in vitro* activity comparable to ciprofloxacin (16). Two 8-fluoroquinolones, however, demonstrated a greater phototoxic potential in a mouse phototolerance model than did reference agents such as ciprofloxacin (17). The toxicity pattern of these congeners was ascribed to the generation of singlet oxygen (18). This photoliability could be removed by replacement of the 8-fluorine by a trifluoromethyl group, as in **5**, with retention of most of the *in vivo* activity (19).

Replacement of the typical nitrogen substituent at C-7 by an sp^2-hybridized carbon resulted in analogs **6** and **7**. **6** showed similar *in vitro* and *in vivo* activity to its nitrogen isostere ciprofloxacin. Despite *in vitro* activity eight-fold inferior to its nitrogen analog **8**, **7** displayed similar *in vivo* activity (20). Another successful application of a carbon-linked C-7 substituent is T-3761 (**9**), which outperformed ciprofloxacin and ofloxacin against a variety of experimental infections (21). T-3761 elevated serum levels of theophylline in rats, but its epileptogenic activity upon intracerebral injection was less than that of ofloxacin or enoxacin (22). Further work on N-1 t-butyl-substituted naphthyridines identified vicinally disubstituted pyrrolidines BMY 41889 (**10**) and BMY 41802 (**11**) as compounds more potent than ciprofloxacin *in vitro* against gram-positive and anaerobic bacteria; their protective ability against gram-positive infections in mice was also superior to ciprofloxacin (23). Investigation of novel quinolone nuclei has continued, with the synthesis of dehydroofloxacin **12** and the fused tricyclic **13**. Both showed disappointing antibacterial activity, however (24,25).

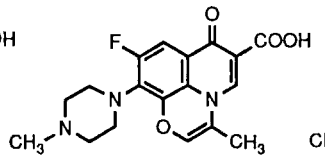


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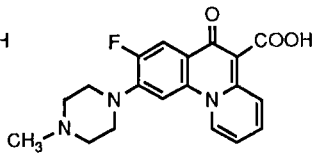


10 R = β-CH_3

11 R = α-CH_3



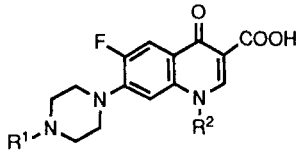
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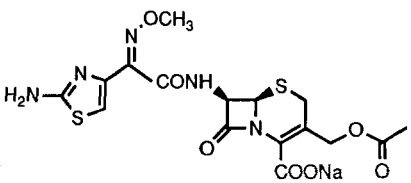
A series of compounds bearing azetidines at C-7 displayed improved activity over ciprofloxacin against gram-positive organisms and anaerobes (26). E-4501 (**14**) was superior to ciprofloxacin against *S. aureus* and *Pseudomonas* infections in mice. In the vicinally substituted azetidine series, the (2S,3R) substitution pattern found in E-4695 (**15**) conferred the best *in vitro* and *in vivo* activity (27). Modification of the 3-methylpiperazinyl substituent found in temafloxacin resulted in the spirocyclopropyl derivative **16**, which exhibited gram-positive activity superior to ciprofloxacin, although its gram-negative activity was somewhat inferior. **16** performed similarly to ciprofloxacin in a mouse infection model with *S. aureus* (28).

New β-lactam-quinolone hybrids were reported; the carbamate-linked Ro 24-4383 (**17**) and amidino-linked **18** show broad spectrum activity and exhibit aqueous solubility much greater than



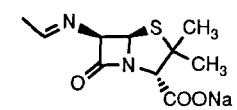
17 R^1 =

R^2 = c-C_3H_5



18 R^1 =

R^2 = Et



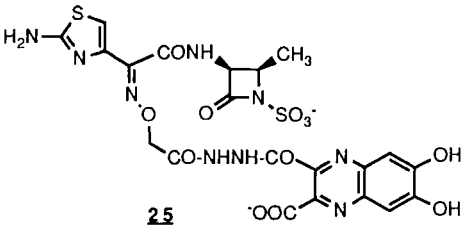
that of the quinolone component alone (29,30). Utilization of an L-amino acid prodrug moiety, as in PD 131112 (**19**) also provided high aqueous solubility and excellent *in vivo* potency in a mouse model (31). Clinical studies with temafloxacin and the *in vitro* and *in vivo* activity of AT-4140 (sparfloxacin) were the subject of numerous posters at the 30th ICAAC (32,33). AT-4140 and WIN 57273 show good *in vitro* activity against *Mycobacterium avium* complex (MAC); WIN 57273 showed a reduction in mortality in a MAC mouse infection model (34). SAR for activity against MAC has been investigated and found not to parallel that for typical antibacterial activity (35,36). Various fluoroquinolones were found to protect the human lymphocyte CEM cell line from HIV-1-mediated cytotoxicity (37). Cinodine, a glycocinnamoylspermidine antibiotic structurally unrelated to quinolones, was found to inhibit *M. luteus* DNA gyrase (38).

Mechanisms of resistance to quinolones have been reviewed (39,40). Cloning of the *gyrA* and *gyrB* genes of *S. aureus* revealed close homology with the respective *E. coli* subunits; some mutational pathways resulting in resistance were therefore suggested to be similar for the two organisms (41). The absence of plasmid-mediated resistance to the quinolones, despite significant selective pressure, has been discussed (42). The clinical emergence of resistance, particularly in MRSA and *P. aeruginosa*, continues to be of concern (43,44).

The B subunit of DNA gyrase has been crystallized in two dimensions (45) and crystals of a truncated A subunit have been diffracted to 4.5 A resolution (46). Studies of this type are facilitated by the availability of strains overproducing the gyrase A and B subunits (47). Neutron and light-scattering studies of gyrase and the gyrase-DNA complex support a structure displaying cavities in the A_2B_2 tetramer, as might be expected considering the DNA strand-passing mechanism of the enzyme (48). The interaction of quinolone antibacterials with eukaryotic topoisomerases has been reviewed (49). A common binding site and mechanism of action are suggested for antibacterial quinolones and antitumor agents, based on studies of the interaction of these compounds with the type II DNA topoisomerase of bacteriophage T4 (50).

β-Lactams - The use of third generation cephalosporins in clinical practice was reviewed (51,52). Research in the cephalosporin area was aimed at obtaining broad-spectrum agents with improved activity against *S. aureus*, especially MRSA, and *Pseudomonas* sp. A new fluoromethoxyimino parenteral cephalosporin, E1077 (**20**), showed at least 2-fold greater activity than cefpirome against MRSA and *P. aeruginosa*. E1077 was highly stable to hydrolysis by various β-lactamases and exhibited a plasma elimination half-life of 1.8 hr in monkeys (53-56). An extensive SAR paper on cephems bearing ammonio-1-propenyl C3 side chains, such as E1077, was published (57). A new parenteral cephalosporin, FK-518 (**21**), with a half-life in the monkey of 1.4 hr, has an MIC_{90} against *P. aeruginosa* four-fold lower than that of ceftazidime; it also exhibited potent activity against both β-lactam- and aminoglycoside-resistant *P. aeruginosa* strains, but was inactive against MRSA (58-60). A new isoxazolidine-containing parenteral cephalosporin, SPD-391 (**22**) was over 30-fold more potent than ceftazidime versus *P. aeruginosa* (61). A paper describing the SAR leading to MT703 and a strategy for incorporating catechol-O-methyltransferase stability to catechol-containing cephalosporins (i.e., replacement of the catechol group with dihydroxypyridone) was published (62). The S-isomer of MT703 (**23**) was found to be slightly more potent than the R diastereomer, retaining an excellent spectrum of activity including *P. aeruginosa* (63,64).

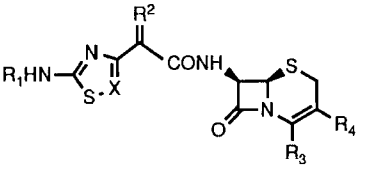
Recently, specific proteins from the *ton*B-dependent iron transport locus, Fiu and Cir, which are involved in the uptake of β-lactam antibiotics bearing catechol or hydroxypyridone groups, have been identified as transporters of monomeric catechol and congeners (65). It was postulated that their main function is the recapture of hydrolytic products resulting from the breakdown of the siderophore enterobactin (66).

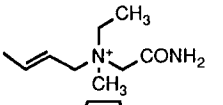
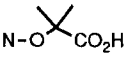
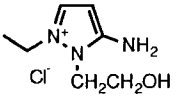
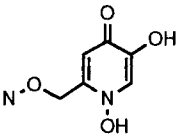
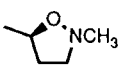
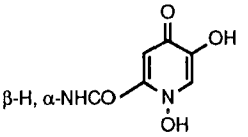
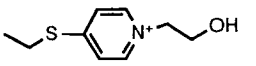
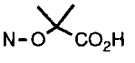
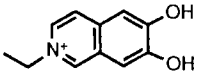
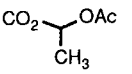
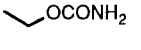


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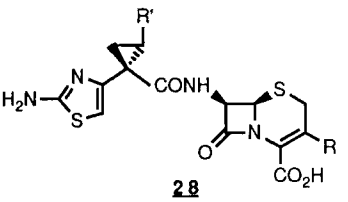
An overview of the use of catecholic substituents to facilitate the transport of β-lactam agents into gram-negative bacteria appeared (67). As with SPD-391 and MT703, the novel antipseudomonal cephalosporin BO-1341 (**24**) exploits the *ton*B-dependent iron transport pathway to penetrate bacteria; BO-1341 is fully active against bacterial constructs lacking OmpC and OmpF (68). Against *P. aeruginosa* isolates from bacteremic and cystic fibrosis patients, BO-1341 had 4-fold greater activity than ceftazidime (69). This strategy is still under investigation in the monobactam area,

where examination of a variety of catechol surrogates led to the synthesis of SQ 33,110 (**25**). Use of the quinoxaline ring system in **25** optimizes both *in vivo* efficacy and urinary excretion (70).



	R^1	X	R^2	R^3	R^4
20	H	N	N—OCH_2F	COO^-	
21	H	N		CO_2H	
22	H	CH		CO_2H	
23	H	CH		COO^-	
24	H	CH		COO^-	
26	H	CH	N—OH		
27	H	CH	$CHCH_2CH_3$	CO_2POM	
29	L-ala	CH	N—OCH_3	CO_2POM	H

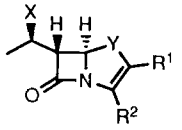
Orally active cephalosporins with improved properties continue to be pursued, but an agent with potent activity against MRSA, *Enterococci*, and *Pseudomonas* sp. has not been identified. YM-22561 (**26**), the acetoxyethyl ester of YM-22508, was predicted to have a long duration of action on the basis of its long half life in dogs and 88% binding to human serum (71). *In vitro*, YM-22508 was found to be similar in potency and spectrum to cefuroxime, cefixime, and cefdinir (72). S-1108 (**27**) is an oral prodrug of S-1006, whose activity against gram-positive and gram-negative bacteria is similar to that of cefteram and cefixime. It showed weak activity against *P. aeruginosa* and *Enterococci* and was inactive against MRSA. S-1006 is highly stable to penicillinase and cephalosporinase enzymes, but unstable to oximinocephalosporinases (73-75). A series of cyclopropylamido cephems **28** were prepared; the cyclopropyl congeners, however, showed decreased *in vitro* potency compared to the vinyl analogs (76). Oral pharmacokinetics in several animal species and mouse *in vivo* efficacy data were described for KY-020 (**29**), a sweet tasting (300x > sucrose) oral prodrug of ceftizoxime (77).

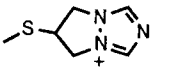
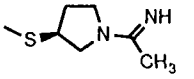
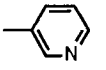
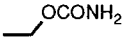


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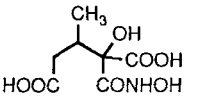
Replacement of the typical hydroxyethyl substituent at position 6 of carbapenems by an aminoalkyl group was investigated; the 1(R)-aminoethyl substituent (as in **30**) was found to impart

the best balance of stability and antibacterial activity (78). Generally, compared to the hydroxy derivatives, the amino analogs displayed improved chemical stability at pH 2, but higher lability at pH 7.4. Introduction of the amino group resulted in reduced activity against gram-positive organisms, but conferred much stronger anti-pseudomonal activity, both *in vitro* and *in vivo*. Unlike the hydroxy series, the 8(R) and 8(S) amino compounds displayed equivalent antibacterial activity, although only the 8(R) compounds were absorbed following oral administration (79,80).



	X	Y	R^1	R^2
30	NH_2	CH-β-CH_3		COOH
31	OH	CH-β-CH_3		COO^-
32	OH	CH_2		COOH
34	OH	S		COOH
35	OH	S		$COOCH_2OCOCH_3$
36	OH	O		COOH
37	OH		CHO	CO_2Allyl

LJC 10,627 (**31**), a broad spectrum carbapenem with high stability to renal dehydropeptidase 1 (DHP-1), exhibited a lower propensity than imipenem to cause convulsions in animals or interfere with GABA binding, and is predicted to show minimal neurotoxic effects (81). Despite inferior MIC's, CS-533 (**32**) was equivalent to or more effective than imipenem against *Pseudomonas* infections in mice (82). The nephrotoxicity induced in rabbits by intravenously administered high doses of CS-533 was effectively blocked by CS-443 (N-benzoyl-β-alanine); this was attributed to inhibition of CS-533 transport into renal tubular cells (83). A Phase I intravenous study using this combination gave a 70 min half-life for CS-533, with 30% urinary recovery after 12 hours (84). The new DHP-1 inhibitor WS1358A1 (**33**) was isolated from *Streptomyces parvulus*, and found to be over 40-fold more potent than cilastatin, as judged by its IC_{50} versus porcine DHP-1 (85). The effectiveness of the two inhibitors *in vivo* is similar (86). The pyridyl-substituted penem **34** exhibited significantly increased gram-negative activity over its phenyl predecessor. **34** was found to possess high stability to DHP-1; its acetoxymethyl prodrug was 67% bioavailable and proved efficacious in mouse infection models (87). Clinical studies of FCE 22891 (**35**), the acetoxymethyl ester of FCE 22101, showed that this oral agent has bioavailability of 32-42% (88,89). The effect of concomitant administration of cilastatin was also investigated (90).



33

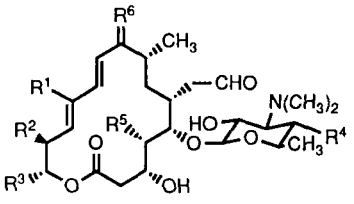
Isopropyl derivative **36** was found to be the most stable analog of several 1-oxapenems that were prepared. These clavulanic acid/carbapenem hybrids exhibited weak antibacterial activity, but potent β-lactamase inhibition, with a spectrum resembling *epi*-thienamycin rather than clavulanic acid (91). The homologous 1-oxacephems containing the hydroxyethyl side chain were reported to be only slightly antibacterial; homothienamycin is known to be virtually inactive (92). Hydroxyethyl cephems were prepared and found to be devoid of antibacterial activity, but potent as β-lactamase inhibitors. The R sulfoxide **37** had activity comparable to sulbactam and clavulanic acid against both TEM penicillinase and various cephalosporinases (93). The synthesis and β-lactamase inhibitory

activity of 6-methylene penems substituted with a monoheteroaromatic ring at C-8 has been described (94); examination of interaction of the previously reported triazole BRL 42715 with β-lactamases, including a cephalosporinase, revealed that the inhibition is stable, with a stoichiometry of inhibition of essentially 1:1 (95). The steady-state half-life and AUC of BRL 42715 were increased upon coadministration with piperacillin; the combination was well tolerated (96).

A review on the mechanism of resistance to β-lactam antibiotics was published (97). Site-directed mutagenesis of class A β-lactamases from *Bacillus* strains and the class C β-lactamase from *Citrobacter freundii* was used to determine the importance of specific amino acid residues to the catalytic cycle (98-100). The three-dimensional structure of a class C β-lactamase, determined by X-ray crystallography, exhibited similarity to the class A β-lactamases, despite limited sequence similarity. A crystal structure of the aztreonam acyl-enzyme derivative allowed the authors to suggest a detailed mechanism for β-lactam hydrolysis (101). BIL-1, a plasmid-encoded β-lactamase which is almost 4000 times more resistant to inhibition by clavulanic acid than is TEM-1, was isolated. This is the first example of a β-lactamase which confers transferable resistance to all penicillins, first, second and third generation cephalosporins, and clavulanic acid (102).

Outer membrane protein D2 of *P. aeruginosa*, underexpression of which leads to imipenem resistance, was purified, and shown to facilitate entry of only those carbapenems and penems which bear a positive charge on their C-2 substituent (103); protein D2 is suggested to recognize basic amino acids and peptides (104). An altered PBP-4 was discovered in an imipenem-resistant *P. aeruginosa* clinical isolate; MICs for a variety of other antibacterial agents were unaffected (105). It was reported that suppression of methicillin-resistance in *S. aureus* by polidocanol ($C_{12}H_{25}(OC_2H_4)_{\sim 9}OH$) occurs *via* inhibition of PBP-2' synthesis (106).

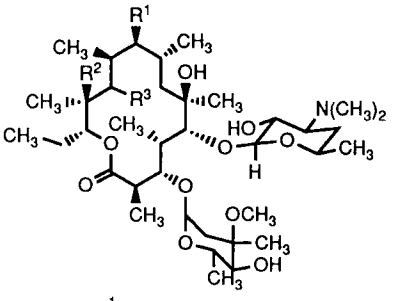
Macrolides - Development of new macrolide antibacterial agents has focused on agents with a broad spectrum of activity including gram-negative (*Haemophilus influenzae*) and MLS-resistant bacteria, high oral absorption, acid stability, and minimal gastrointestinal side effects. Azithromycin (107) and



	R^1	R^2	R^3	R^4	R^5	R^6
38	CH_3	CH_2OMycn	Et	PMB-Myc	CH_3	O
39	H	H	CH_3	iVal-Myc	OCH_3	α-$N(CH_3)_2$
40	CH_3	CH_2OAc	Et	H	CH_3	α-$N(CH_3)_2$

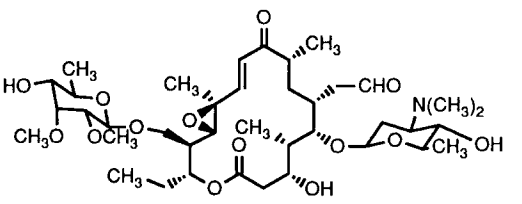
iVal-Myc = 4-O-isovaleryl-mycarosyl PMB-Myc = 4-O-paramethoxy-benzylmycarosyl Mycn = 2,3-di-O-methyl-mycinosyl

clarithromycin are recent examples of second-generation macrolides that come close to fulfilling these goals (108). Twenty-four posters were presented at the 30th ICAAC meeting on the clinical evaluation of clarithromycin, including a report on its effectiveness against MAC in AIDS patients (109). Semisynthesis of 16-membered ring macrolides has yielded promising, clinically significant results in overcoming MLS-resistance. YM133 (**38**) is a tylosin derivative with improved *in vitro* and *in vivo* activity against macrolide-resistant clinical isolates of *S. aureus* (110,111). However, YM133 had decreased potency against *E. coli* when compared to erythromycin. Synthesis of 9(R)-N,N-dimethylamino-9-deoxo derivatives of niddamycin (**39**) and 23-O-acetyl-4'-deoxy-5-O-mycaminosyl-tylonolide (**40**) was undertaken. The allylic 9(R)-N,N-dimethylamino group was found to be optimal. Analogs **39** and **40** showed some improvement in *in vivo* performance over erythromycin (112). The 9-N-decyl derivative of erythromycylamine (**41**) and the 12,13-epoxy-2'-deoxy desmycosin derivative **42** inhibited *S. epidermidis* strains constitutively expressing MLS resistance (113). A new erythromycin A derivative, A-69334 (**43**), showed similar activity to erythromycin *in vitro*, but superior oral activity versus *H. influenzae* in mouse models (114). A-69334 had a plasma half-life of >6 h in the monkey and was twice as bioavailable as erythromycin A and azithromycin (115). In addition, A-69334 accumulated in human PMN's to a greater extent than erythromycin A and showed a significantly reduced GI smooth muscle stimulating activity (116,117). An efflux mechanism of resistance specific for 14-membered ring macrolides and type B streptogramins was identified (118,119).



41 $R^1 = NH(CH_2)_9CH_3$
$R^2 = OH$; $R^3 = \beta$-OH

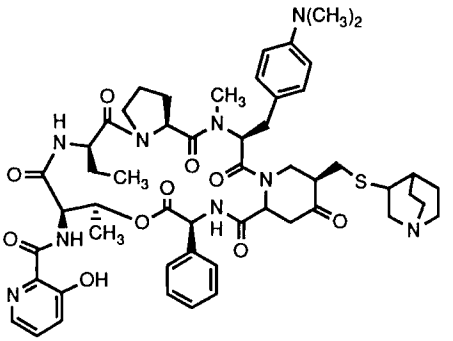
43 $R^1, R^2 =$ 
$R^3 = \alpha$-NH_2



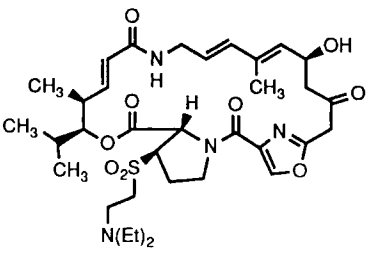
42

Glycopeptides - The mechanism of resistance to glycopeptides is under investigation (120-123), as the emergence of resistance to vancomycin and teicoplanin is becoming an increasing problem clinically (124-126). This has also prompted the search for new analogs with activity against resistant organisms, especially coagulase-negative staphylococci (127,128). Analogs of teicoplanin were prepared by condensation with 3,3-dimethyl-1-propylamine; the resulting amide, MDL-62,873, had increased activity against coagulase-negative staphylococci while maintaining the spectrum of teicoplanin against susceptible strains (129,130). Several N^{15}-alkyl and N^{15},N^{15}-dialkyl analogs of teicoplanin were synthesized, but the alkylated analogs did not show marked advantages over teicoplanin (131). A review on teicoplanin antibacterial activity, pharmacokinetics, and use in therapy appeared (132). Solution structures of vancomycin and a vancomycin/D-ala-D-ala complex were obtained from 2D NMR experiments (133).

Miscellaneous - A number of papers at the 30th ICAAC meeting were devoted to magainins, peptide antibiotics isolated from frog skin. These communications focused on the lack of resistance development to the magainins after several serial passages, as well as *in vitro* potency and spectrum, particularly against organisms that are resistant to current therapy, such as MRSA, gentamicin-resistant *P. aeruginosa*, mycobacteria and amoebae (134-136). Magainins composed entirely of D-amino acids were synthesized and found to retain antibacterial potency, but resist proteolytic cleavage (137). RP59500 is a synergistic, water-soluble combination of RP57669 (44) and RP54476 (45), which are semisynthetic derivatives of the two main components of the naturally occurring streptogramins. This family of antibiotics inhibits protein synthesis by interfering with ribosomal function. The combination was found to be active against methicillin-resistant and erythromycin-resistant *S. aureus* and *S. epidermidis*, with MIC_{90}'s of 1.0 and 0.5 µg/ml. RP59500 had MIC_{90}'s comparable to clarithromycin and azithromycin against *Streptococcus*, and was selected as a candidate for further clinical studies for the IV treatment of serious infections (138). Benzoxazinorifamycin analogs were specifically developed for use in treatment of MAC, typically



44



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encountered in AIDS patients. These analogs were 8- to 15-fold more active than rifamycin against various mycobacteria sp. and less toxic in mice. KRM-1648 was the most efficacious analog against a *M. intracellulare* infection in mice (139). The orally bioavailable 2'-(N,N-diethylamino) derivative of rifamycin P also showed good activity against MAC, with an MIC_{90} of 2 μg/ml (140).

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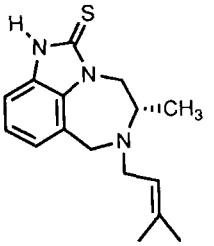
Chapter 14. Antiviral Agents

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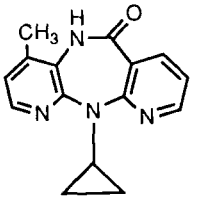
Introduction - This chapter will provide an overview of developments in the antiviral field over the last year, with a concentration mainly upon Human Immunodeficiency Virus (HIV) and the Herpes Simplex Virus (HSV) family of viruses. Emphasis is given to recent clinical results. The major effort in antiviral chemotherapy continues to be directed to Acquired Immunodeficiency Syndrome (AIDS).

Retroviruses - Several reviews have been published over the last year concentrating upon antiviral treatment of HIV infection (1-5). In this section, we will examine approaches to AIDS drug development with the exception of HIV protease inhibitors which is the subject of Chapter 15. Novel retroviral targets are discussed in Chapter 18.

Non-nucleoside inhibitors of the key viral enzyme reverse transcriptase (RT) continue to be identified, of which R-82150 (1) is one of the most selective and potent inhibitors of HIV-1 *in vitro* (6). More recently the dipyridodiazepine 2 (BI-RG-587) has also been shown to be a potent inhibitor of HIV-1 (IC50=42 nM) *in vitro* (7), with an exquisite selectivity for HIV-1 over HIV-2 and other retroviruses; it shows synergy with AZT and is active against AZT-resistant HIV-1 isolates *in vitro* (8). The Merck compound L-697,639, another non-nucleoside inhibitor of RT, is in early clinical trials; at present no chemical structure is available for L-697,639 (9).



1



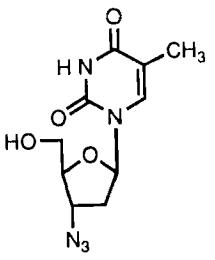
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AZT (3, zidovudine) remains the only approved therapy for HIV infection (10). Two clinical trials have addressed the possibility of using lower doses and demonstrated that zidovudine (300 - 1000 mg/day) was as effective in treating advanced AIDS as the original recommended dose of 1500mg/day (11,12). The lower doses were less toxic with no differences seen in either the time of development to another opportunistic infection, or change in CD4 count. After 24 months no differences were seen between the lower doses and the standard treatment (11). In a study with mildly symptomatic and asymptomatic patients, lower doses helped delay progression of HIV disease and produced little toxicity in the patients (13-15). However, zidovudine fails to prevent onset of HIV infection even when given within 45 minutes post exposure to the virus (16). Patients with symptomatic HIV infection who have survived for three years or more on zidovudine may have a higher probability of developing non-Hodgkins lymphoma (17). Resistance to AZT is related to a number of factors, including stage of the disease and the dose administered, but it should be stated that while resistance has been found *in vitro*, the clinical implications of this finding are still unclear (18). While AZT-resistant isolates show cross resistance to only those analogues bearing a 3'-azido group (19), the isolates remain sensitive to other nucleoside analogues. A review on zidovudine intolerance has also been published (20).

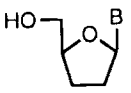
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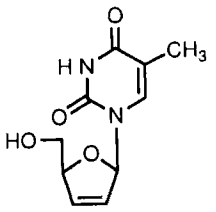
The preclinical development of dideoxyinosine (ddI, Videx, **4**) has been reviewed (21). The early clinical results suggest that ddI can be can be taken orally if given with an antacid, with a maximum tolerated dose of 12 mg/kg/day. The recommended dose appears to be about 9.6 mg/kg/day. The major toxicities seen are either peripheral neuropathy and/or pancreatitis (22-27). Since the dose limiting toxicity with ddI is different from AZT, there appears to be a potential role for ddI as a therapy for HIV infection. Peripheral neuropathy was also the dose limiting toxicity observed earlier with dideoxycytidine (ddC, Hivid, **5**) (28). More recent studies in pediatric patients show that ddC can be taken for short periods or in combinations with AZT and produce a beneficial effect (29). A dose escalation study has been performed in HIV-infected patients with 2',3'-didehydro-2',3'-dideoxythymidine (d4T, **6**). Dose limiting toxicity was again peripheral neuropathy, which was slow to onset and was reversible upon discontinuation of the drug (30-32). Reports continue to appear which outline the synergy of AZT with various other compounds such as interferon α (33,34), ddC (35) and ddI (36). A review on the clinical application of AZT and related dideoxynucleosides has been published (37).



3



4 B = HX
5 B = C



6

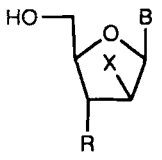
Two other 3'-azido analogues, 3'-azido-2',3-dideoxyuridine (AZU, **7**) and 3'-azido-2',3'-dideoxyguanosine (AZG, **8**) show good anti-HIV activity. AZU has been found to have essentially the same pharmacokinetics as AZT (38, 39). There is extensive degradation of AZG, with only 10 % of the radiolabelled compound found as the AZG nucleotides; AZG-DP was the predominant nucleotide at all time points suggesting that it is the nucleoside diphosphate kinase that is the rate limiting step in the formation of AZG-TP (40). The 2'-azido and 2'-amino analogues of 3'-deoxythymidine and 2',3'-dideoxyuridine were inactive (41-43). Introduction of most groups at the 3'-position of either 3'-deoxythymidine or 2',3'-dideoxyuridine gave inactive compounds (44, 45). Thus, the substitution pattern permissible on the ribose moiety, in order to retain activity, is clearly limited.

3'-Fluoro-2',3'-dideoxythymidine (FddT, **9**) has also been reported to have good anti-HIV activity with similar pharmacokinetics to AZT in the monkey (46). However, FddT was found to be considerably more toxic than either AZT or d4T to mice (47). 5-Chloro-3'-fluoro-2',3'-dideoxythymidine appears to be the most promising of the 5-substituted analogues (48, 49). The 2'-fluoro analogue 2'-fluoroarabino-2',3'-dideoxyadeonsine (**10**, FddA) has comparable potency to ddA but is deaminated 15 times slower than ddA by adenine deaminase (50). The mechanism of action of FddA is similar to that of ddA except that a greater proportion of the active triphosphate species, FddA-TP, is obtained by direct phosphorylation of the free nucleoside (51). The corresponding 2'-fluoro-2',3'-dideoxycytidine analogue (**11**, FddC) was also active in several different cell lines; it's potency is approximately 10 % that of ddC (52-54).

The pharmacology of dideoxyguanosine (**12**, ddG) suggests that the antiviral activity of ddG in MT-2 cells (which is comparable to ddA) is not affected by either the addition of guanosine or deoxyguanosine (55). This result is given further credence by reports that the initial phosphorylation of ddG is catalyzed by a phosphotransferase (5'-nucleotidase) enzyme which utilizes inosine monophosphate (IMP) as the phosphate donor (56). Ribavirin has also been shown to potentiate the activity of ddI and 2',3'-dideoxy-2,3-diaminopurine riboside in MT-4 cells (57).

Several halogenated congeners of 2',3'-dideoxypurine nucleosides have been prepared in order to improve pharmacokinetic properties (58). The 8-hydroxy compound, 2',3'-dideoxy-8-hydroxyadenosine, was resistant to glycosidic cleavage by phosphorylases (59). The N^6-methyl

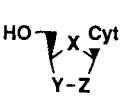
derivative (**13**) had comparable activity to ddA, but was not deaminated by adenosine deaminase. Interestingly, the corresponding 2'-fluoro analogue (**14**) was considerably less potent than FddA, while the d4A analogue was also inactive against HIV (60).



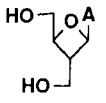
7 R = N_3; X = H; B = U
8 R = N_3; X = H; B = G
9 R = F ; X = H; B = T
10 R = H ; X = F; B = A
11 R = H ; X = F; B = C
12 R = H ; X = H; B = G
13 R = H ; X = H; B = N^6-Me-Adenine
14 R = H ; X = F; B = N^6-Me-Adenine

A limited number of 2',3'-didehydro-2',3'-dideoxynucleosides have been prepared. Several papers outlining synthetic approaches to these series of compounds have appeared (61-62). In addition, two groups have published on various esters or analogues as prodrugs of d4T (63-64). Introduction of a nitrogen into the 6-position of d4T or d4U rendered the nucleoside analogues inactive (65). A review of the structure-activity relationships of these dideoxynucleosides has been published (66). Of the unusually substituted dideoxynucleosides, BCH-189 (**15**) is scheduled for clinical trials (67). The new related analogues **16** and **17** have recently been reported and are inactive against HIV (68, 69).

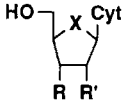
3'-Homologated analogues have recently received considerable attention, much of this based upon the finding that oxetanocin A (**18**) has broad antiviral activity (70). Both of the ddA analogues with a hydroxymethyl substituent at either the 3'- or 2'-positions (**19** and **20** respectively) were less active than ddI; however, **19** was superior to **20** at 10 μM (71).



15 X = O ; Y = S ; X = CH_2
16 X = CH_2 ; Y = S ; Z = CH2
17 X = CH_2 ; Y = CH_2 ; Z = O

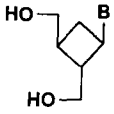


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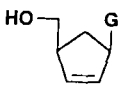


19 R = CH_2OH ; R' = H ; B = A
20 R = H ; R' = CH_2OH ; B = A

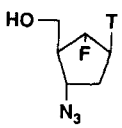
The carbocyclic analogues of oxetanocin retain broad spectrum of antiviral activity against HIV, HSV-1, HSV-2 and cytomegalovirus (CMV) (72-75). Amongst these, cyclobut-G (**21**) and cyclobut-A (**22**) were the most potent with an ID50 = 1 μM against HIV. While the effect of **21** can be reversed by the addition of 2'-deoxy-guanosine, 2'-deoxyadenosine does not reverse the antiviral effect of **22** (72). All the HSV activity of **21** resides in the (+) isomer, yet it is the (-) isomer that is phosphorylated more readily by thymidine kinase (75). Both **21** and **22** have similar *in vitro* toxicities to AZT on CFU-GM (72). Carbovir (**23**) is another carbocyclic nucleoside analogue which has shown good *in vitro* activity against HIV (76, 77). Carbovir also exerts its biological effect once metabolized to the triphosphate analogue (77). The carbocyclic analogue of AZT in which the oxygen of the furan ring has been replaced by CHαF, **24**, is much less effective as an anti-HIV agent than AZT (78).



21 B = G
22 B = A

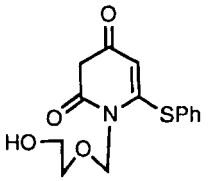


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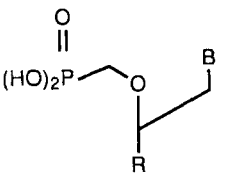


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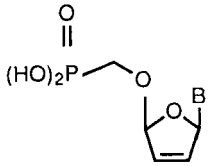
The acyclic nucleoside derivative HEPT (**25**) was found to be active against HIV-1, but it was not active against HIV-2 (79). The acyclic nucleotide analogue PMEA (**26**) had previously been shown to be active in its own right and was synergistic with AZT against HIV (80). Recent results indicate that PMEA was more effective than AZT *in vivo* in a Moloney murine sarcoma virus model (81). In the feline systems, PMEA at 20, 5 or 2 mg/kg given intramuscularly, showed a dose dependant suppression of FIV replication (82). PMEA may be unique in that, in the murine sarcoma model, it was more effective when administered as a single dose on the day of the infection than when given 2, 4 or 7 times over the course of the week (83). This potential for infrequent dosing is essential, given that the oral bioavailability of PMEA is rather low (81). The d4A- (**28**) and d4T-phosphonate (**29**) are also very interesting compounds (84). D4A-phosphonate (**28**) is remarkably potent against Rauscher murine leukemia virus (R-MuLV) with an ID50 = 0.003 μM, and an ID50 = 1.5 μM against HIV. Several alternative monophosphate analogues such as **30** and **31** were prepared, and only these two showed any appreciable activity (85).



25



26 R = H ; B = A
27 R = CH_2OH ; B = C



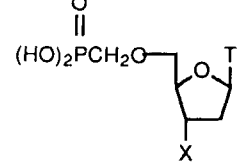
28 B=A
29 B=T

Other Targets - The crystal structure of the soluble CD4 binding fragment has been published with 2.3 angstrom resolution (86, 87). The structure consists of two intimately associated immunoglobulin-like domains, with the residues implicated in recognition being in domain 1. Several companies continue to evaluate TAT inhibitors as an approach to control of HIV I infection. 2,3-Dimercapto-1-propanol was reported to inhibit HIV-1 activity, virus production and infectivity *in vitro* (88). The 6-O-butanoyl derivative (MDI 28,374) of castanospermine, a potent glycosidase inhibitor, had previously been shown to have a better therapeutic index *in vitro* than the parent compound (89). However, against Ranscher murine leukemia virus, no difference could be detailed between the two compounds. While effective, both castanospermine and the 6-O-butanoyl derivative were less effective than AZT in this model system (90).

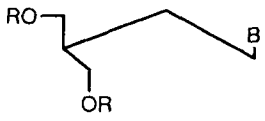
Herpesviruses - A large amount of nucleoside and nucleotide analogue work continues to focus on herpesviruses. New trends include greater effort on the inhibitors of the viral thymidine kinase (TK) and on the design of prodrugs. The potential for inhibitors of TK to have long term therapeutic benefit by suppressing reactivation of the latent phase has been reviewed (91). TK is not essential for virus replication in tissue culture, therefore practical evaluation depends upon the development of models for latency; some progress has recently been made with this approach. N^2-Phenyl-2'-deoxyguanosine and N^2-m-(trifluoromethylphenyl)guanine were shown to reduce the frequency of reactivation of virus from explant cultures of latently infected murine trigeminal ganglia (92). Structure-activity data reported for a number of N^2-substituted guanine derivatives led to the identification of the above mentioned m-trifluoromethylphenyl analogue as the most potent inhibitor of TK (93). Another substance, (±)-[(Z)-2-(hydroxymethyl)cyclohexyl)methyl] guanine, suppressed reactivation *in vitro* (94). A series of 5-arylamino-substituted deoxyuridine derivatives are reported to be very potent inhibitors of HIV-1 TK with potencies in the nanomolar range. This is compared with estimated IC50 values > 200 μM against two human cellular TKs (91,95).

Studies have continued on new and older nucleoside analogues, primarily guanine derivatives. A detailed review of penciclovir (**32**) and derivatives was published (96). Also, the metabolism of prochiral pencyclovir was studied by the synthesis of a chiral ^{13}C-derivative. The (S)-isomer was shown to be produced in HSV-1 infected cells (97). Famciclovir (**33**), a prodrug of **32**, was shown to give enhanced oral bioavailablity in the rat, and metabolism data were correlated with human tissue

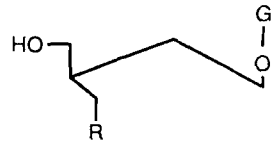
(98). The 2-hydroxymethyl isomer of **32** was shown to be the most selective of a series of compounds tested in tissue culture against human herpesvirus 6 (99), but had lower oral bioavailability than famciclovir in rats and monkeys (100). Another isomer, the 3,5-dihydroxypentyl analogue, showed limited activity against HIV-1 (101). Isomers of acyclovir and ganciclovir that involve attachment of the side chain via an oxygen atom directly to the N-9 position, compounds **34** and **35**, show potent activity against HSV and VZV (102). Also, in this series the 9-(2,3-dihydroxypropoxy) derivative was quite active (103), while the adenine and pyrimidine analogues were inactive (104).



30 X=N_3
31 X=F



32 R=H ;B = G
33 R=Ac; B = 6-deoxyguanine

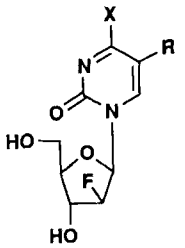


34 R=H
35 R=CH_2OH

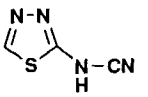
Further studies on phosphonomethyl nucleotide analogues led to the identification of HPMPC (**27**) as a lead compound, which is especially potent *in vivo* against herpes simplex virus (105). This substance has also been shown to have *in vivo* activity in murine cytomegalovirus and simian varicella virus models; it has the potential for less than daily dosing to achieve efficacy (106). Mechanistic studies have demonstrated that phosphonomethyl nucleotide derivatives inhibit both the DNA polymerase (107) and ribonucleotide reductase (108). In the guanine series, structure-activity studies have been published (109, 110). The close structural analogues of acyclovir and ganciclovir monophosphate show good *in vitro* activity against herpesvirus (111, 112).

While acyclovir-resistant HSV from immunocompetent patients is uncommon, recent studies show that in immunocompromised patients, resistance arises fairly frequently. (113). Of concern is the finding that the combined toxicities of ganciclovir and AZT are such that it may necessitate discontinuation of one of the drugs (114). Various combinations of DHPG with other drugs to modulate the toxicity were reported; foscarnet appears to act synergestically with DHPG (115, 116).

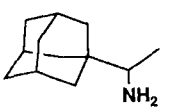
Other Viruses - The ongoing search for safe substances which have the potential for suppression of hepatitis B virus (HBV) replication by chronic administration has led to the identification of deoxyguanosine, FddT (**9**) and phosphonate nucleotide analogues such as HPMPA as potential anti-hepatitis agents (117-119). *In vivo* activity against HBV was demonstrated for 2,6-diaminopurine-2',3'-didexyriboside (120), 1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl)-5-ethyluracil (**36**, FEAU) (121) and HPMPA (122). Interferon α-2b has provided indications of clinical efficacy (123). The related fluoroarabino analogue, 1-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl)-5-iodocytosine (**37**, FIAC) has entered clinical trials for CMV; FIAC has the advantage over current CMV therapies in that it can be administered orally (124).



36 X = OH ; R = Et
37 X = NH_2; R = I



38



39

Influenza is still largely untreated and represents a major therapeutic challenge. In extended *in vitro* studies LY 253963 (**38**) was found to have poor selectivity (125). Low doses of rimantadine were

well tolerated and effective when given prophylactically (126). However, clinically significant virus resistance to rimantidine (**39**) has been found, and this substance was not active against the influenza B virus strain (127).

Conclusion - Antiviral chemotherapy continues to be a very active research area with AIDS being the major target. The last two years have seen a change of direction away from nucleoside analogues for HIV towards protease inhibitors and non-nucleoside inhibitors of RT. While the exquisite selectivity of these non-nucleoside inhibitors is impressive, the findings that HIV-1 isolates are sensitive and HIV-2 isolates are not, would suggest that reduced sensitivity over time will also be a problem with this class of compounds. The trend towards other targets such as TAT and RNAse H (the X-ray structure for the HIV RNAse H has recently been published [128]) will continue and increase as more information becomes available. If it transpires that TK inhbition can truly limit reactivation, this will also become a major target for HSV therapy. The need (and trend) towards finding and developing selective and non-toxic agents for specific viruses such as CMV, hepatitis B, influenza, rhinovirus, etc. provides several opportunities in this area.

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Chapter 15. HIV Protease Inhibitors

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Introduction - The catastrophic dimensions of the HIV/AIDS pandemic will be manifest in the next decade. By the year 2000, the World Health Organization projects that the cumulative number of AIDS cases will reach 6 million (1). Fewer than one million of these cases occurred prior to 1990, and less than 25% of the 5 million new cases are considered preventable. Concomitantly, the world-wide prevalence of HIV infection is expected to rise to about 18 million. In the US alone, 1 to 1.5 million people are believed to be already infected with HIV, and, by the end of 1990, close to 100,000 deaths will have been attributed to AIDS (2).

The basic hypothesis that specific anti-retroviral agents should ameliorate the course of HIV infection is now supported by clinical trials with nucleoside-based inhibitors of the viral reverse transcriptase (3). Unfortunately, the most widely used of these agents, AZT and DDI, can have significant toxicity, and mutations in the viral target can lead to drug resistance (4). While non-nucleoside inhibitors of the reverse transcriptase may overcome some of these problems, inhibitors of other essential viral proteins should also be useful, either as single agents or in combination (5-8). Just six years after the sequence of the HIV genome became available, inhibitors of one such protein, the HIV protease, are close to being used in experimental AIDS therapy. Several factors contributed to this rapid progress. Sequence homologies of HIV-1 with previously studied retroviruses supported the existence of the protease enzyme, and the presence of a conserved Asp-Thr-Gly sequence advocated membership in the aspartyl protease class. This classification, coupled with homology based predictions of substrate sequences, meant that the strategies developed for the design of inhibitors of renin, an aspartic proteinase involved in the regulation of blood pressure, would be directly applicable to the HIV protease (9).

Viral Proteases - The replication of numerous plant and animal viruses depends on the specific action of virus-encoded proteinases (10-13). In contrast to their eukaryotic host organisms, these viruses, primarily (+)-RNA viruses and retroviruses, initially express their structural proteins and enzymes as large, multi-domain polyproteins. The assembly of infectious progeny virus from these precursors requires proteolysis of specific peptide bonds between the protein domains. Although the existence of viral polyproteins had been known for over 20 years, the first virus-encoded proteinase responsible for their cleavage was not identified until 1977 (10,14). The potential utility of protease inhibitors as antiviral agents was readily appreciated (15). Fortuitously, the first proteinase to be identified was a structural component of the Rous sarcoma virus, a retrovirus, and the early studies of this and other non-human retroviruses provided an experimental and conceptual framework for the identification of the HIV proteinase as a virus-specific therapeutic target (16).

The HIV-1 Protease - Early predictions regarding the existence, amino acid sequence, three-dimensional structure, and role in viral replication of an HIV proteinase (17-20) culminated in structural proof by X-ray crystallography (21-23). Fundamentally, the HIV proteinase is a C_2 symmetric homodimer in which each of the identical 99 amino acid subunits contributes a single aspartyl residue to the catalytic site (Figure 1). The analogy of this structure to the bilobal domains of mammalian and fungal aspartic proteases has suggested that the retroviral protease may be a genetic "fossil" (20,24). As required by the accepted catalytic mechanism of aspartyl proteases, the two acid residues are juxtaposed about the C_2 axis of the diad (25-29). Consequently, the scissile bond of peptide substrates must nearly intersect with this symmetry element. These axiomatic spatial relationships, coupled with basic transition state theory, have served as a foundation for the design of one class of potent inhibitors of the protease (*vide infra*).

Role of the HIV Protease in the Viral Life Cycle - HIV protease is uniquely responsible for the post-translational cleavage of the viral *gag* and *gag-pol* polyproteins (8). An eighth cleavage site, generating the p51/p66 reverse transcriptase heterodimer, was confirmed by amino acid composition and *in vitro* processing of a model substrate (30). Analysis of the amino acids flanking

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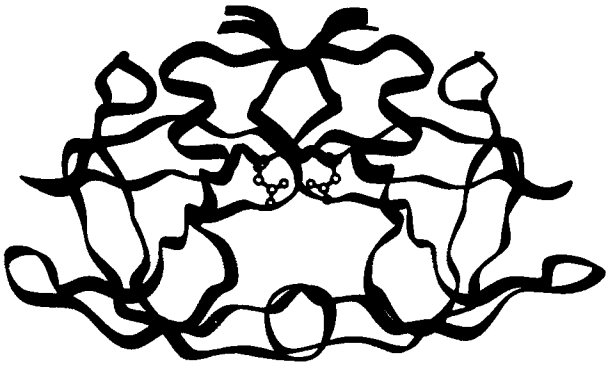


Figure 1. Ribbon diagram of HIV-1 protease, showing active site and catalytic aspartate groups (75).

the scissile bond (GlyAlaGluThrPhe/TyrValAspGlyAla) reveals a symmetrical disposition of large hydrophobic, branched, charged, and small side chains. While evidence of latent C_2 symmetry can be extracted from analysis of the other seven substrates, the determinants of substrate specificity for the protease remain obscure. However, one can speculate that the heterogeneity of the cleavage sites, and consequent differences in cleavage rates, may play an important role in the regulation of viral assembly (31-33). Although site specific mutagenesis of the protease's catalytic aspartate residue to glutamine or alanine does not prevent viral assembly and budding, these mutants retain their immature morphology and are non-infectious (34-36). In electron micrographs of budding wild-type virus, the ring-shaped nucleoids characteristic of immature virus particles appear to be quickly converted to a condensed, bullet shaped core (37,38). These observations are consistent with protease activation within the viral particle. Premature proteolysis in the cytoplasm would remove the N-terminal myristoyl glycine residue from the *gag* and *gag-pol* polyproteins and presumably impair the ability of the viral proteins to aggregate at the cell membrane (39-41). Although the protease is active in free solution, the K_m's for model substrates are in the millimolar range (8). The protease would therefore be expected to be most efficient at the high substrate concentrations enforced by the immature viral particle. X-ray crystallographic studies indicate that generation of free protease *via* autoproteolysis of a *gag-pol* dimer is an unlikely process. Thus, the rate limiting step in viral maturation may be the excision of the protease monomer from a trimeric *gag-pol* polyprotein formed within the budding virus particle. In this scenario, inclusion of protease inhibitors within the viral capsid would be essential for antiviral action.

Evidence continues to accumulate that viral infectivity is dependent on normal processing at several of the defined cleavage sites. In the absence of a competent protease, the viral reverse transcriptase has either low activity or is enzymatically inert (34,36). Mutations at the cleavage sites producing the matrix protein p24 caused aberrations in virion morphogenesis and prevented virus replication (39). In addition to the proteolytic events late in the viral life cycle, additional processing may occur in the early phases of replication. Capsids isolated from the genetically related equine infectious anemia retrovirus suffered pepstatin sensitive cleavage of the nucleocapsid protein p11 to p6 and p4 (42). Cleavage of synthetic substrates confirmed the association of the viral protease with the capsid.

There is limited evidence that cleavage of host proteins may also play a role in the retroviral life cycle or pathogenicity. Reasonably efficient cleavage of intermediate filament proteins by the HIV-1 protease has been observed *in vitro* (43). These proteins are components of the mammalian cell cytoskeleton, and microinjection of protease into human fibroblasts caused alteration in filament distribution and cell morphology. Protein sequencing and cleavage of a synthetic substrate defined one cleavage site as Ser-Ser-Leu-Asn-Leu/Arg-Glu-Thr-Asn-Leu. The presence of an arginine residue in the P_1' position was initially quite suprising since all of the virally encoded substrates possess a hydrophobic amino acid at this site. However, several unique cleavage sites have recently been identified in calcium free calmodulin in which charged amino acid residues occur in both the P_1 and P_1' positions (44).

In Vitro Assays for Protease Inhibition - In addition to traditional HPLC methods, inhibition of substrate cleavage has been assessed with direct radio-, spectrophoto-, and fluorometric techniques (45). Of these, the continuous fluorometric assays offer the versatility needed for both

detailed kinetic studies and large scale screening in a microtiter plate format. In the most sensitive system, complete cleavage of the intramolecularly quenched fluorogenic substrate, DABCYL-Gaba-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS, generated a 40-fold increase in fluorescence intensity (46). Under conditions where the concentrations of both the substrate and inhibitors are much greater than that of the enzyme, the following relationship holds: $K_i = IC_{50}/1 + (S/K_m)$. Thus, direct comparisons of the IC_{50} values obtained with different substrates are only valid when the substrate concentrations are much less than their K_m's. In addition, the actual K_i for a given inhibitor may also vary with pH and salt concentration.

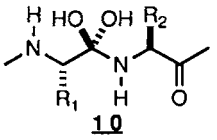
Substrate Specificities - Recent studies have focused on subsite specificity of small peptides based on the known cleavage sites for the HIV protease (Table 1). Lipophilic, β-unbranched amino acids are tolerated in the P_1 subsite (Xaa) of chromogenic substrates **1** but Val, Ile and charged residues are not (47-49). In the P_1' subsite of the *gag* sequence **2**, replacement of proline with pipecolinic acid provided an inhibitor (K_i = 1.4 μM) (50). Perhaps most interesting is the specificity of the P_2 and P_2' subsites, wherein substitution with a variety of residues does not significanty alter the binding constant, but can dramatically affect the rate of catalytic turnover. The k_{cat} values for **3** (Xaa = Asp, Asn) were found to be substantially lower than for **3** (Xaa = Val, Ile, Leu, Ala) while the K_m's were comparable. Similarly, the k_{cat} of substrates **4** diminishes (Xaa = Val, Ile > Leu, Ala > Phe) without a significant change in K_m. Dramatically, mutation of the P_2 Asn residue of *gag* sequence **2** to Gln totally abolishes proteolytic processing (33). Moreover, in accord with the C_2 symmetric nature of the HIV protease active site, substantial reduction of k_{cat} was also observed in the P_2' subsite of **5** (Xaa = Ile > Leu, Ala) with little variation of K_m. There is considerable tolerance for variation in the P_3 subsite of sequences **6**, with processing observed upon substitution with basic, acidic and lipophilic residues. Clearly, the subtle interactions which affect subsite specificity of substrates are not well understood; indeed, the subsite specificities for different cleavage sequences do not parallel each other (33). Processing of nonviral proteins has been investigated with the purpose of defining new substrate sequences for use in inhibitor design. Cleavage of a truncated *Pseudomonas* exotoxin occurs not at the expected Tyr-Pro sequence **7** but at sequences **8** and **9**, a result which has been confirmed using small peptides. Specific processing of denatured ribonuclease A and the ubiquitous mammalian proteins vimentin and calmodulin (*vide supra*) by HIV-1 protease have also been observed (43,44,51). As a caveat, it should be noted that for other aspartyl proteases, variations in k_{cat} have resulted from differences in the rate limiting step of product release, rather than differences in the rate of amide hydration (28). If this holds for HIV protease, examination of k_{cat}/K_m for a series of substrates would have little predictive value for the design of inhibitors which are true transition state analogues.

Table 1. Subsite specificity in substrates of HIV protease.

No.	Sequence	Ref.
	—P_6 — P_5 — P_4 — P_3 — P_2 — P_1 –: —P_1' –P_2' –P_3' –P_4' –P_5' –	
1	H-Lys—Ala—Arg—Val—Xaa–:–Nph[a]-Glu—Ala—Nle—(Gly)–NH_2	(47,48)
2	H–Val—Ser—Gln—Asn–Tyr–:—Pro—Ile—Val—Gln—NH_2	
3	H-Lys—Ala—Arg—Xaa—Nle–:–Nph[a]-Glu—Ala—Nle—NH_2	(48)
4	H–Ala—Thr—His—Gln—Xaa—Tyr–:–Nph[a]–Val—Arg–Lys—Ala—OH	(49)
5	H-Ser—Gln—Asn–Tyr–:—Pro–Xaa—Val—OH	(52)
6	H–Ala—Thr—His—Xaa—Val—Tyr–:–Nph[a]–Val—Arg–Lys—Ala—OH	(49)
7	–Leu–Glu—Arg—Asn–Tyr–X–Pro–Thr—Gly—Ala—	(53)
8	–Ser—Gly—Asp–Ala—Leu–:—Leu–Glu—Arg–Asn–	(53,54)
9	H–Ala—Asn–Leu–:—Ala—Glu—Glu—Ala—Phe–	(53,54)

[a] Nph = *p*-nitrophenylalanine

Design of Protease Inhibitors - Generation of a high energy, tetrahedral diol (**10**) from a low energy, trigonal amide consitutes the key step in aspartic proteinase catalysis (25-29). Although aspartic proteinases can be inactivated by high concentrations of simple epoxides, the absence of an acyl enzyme intermediate in normal catalysis would be expected to severely constrain approaches based on chemical alkylation. So far, attempts to design irreversible inactivators of the HIV protease have yielded only weak inhibitors (55,56). Far greater success has been realized with competitive inhibitors designed to mimic the transition states leading to **10**, or, more accurately, to act as collected substrate analogs (28). The basic principles of this approach were established previously with other aspartic proteinases, such as renin, an important target for antihypertensive therapy (9). The results of these earlier studies have been applied to the HIV protease, both directly, by the screening of known inhibitors of renin



10

or pepsin, and indirectly, by the incorporation of known transition state analogues into the scissile site of retroviral substrates. As previously observed with renin, reduced amide transition state analogues such as **11** - **14** possessed only modest potency. This weakness may stem from the apparent lack of a direct interaction of the inhibitor with the catalytic aspartic acid residues (57). Although significant potency has been reported for acetyl-pepstatin (**15**), introduction of statine or the statine analog AHPPA into HIV-1 cleavage sequences has again provided only modest inhibitors (**16** - **18**). Incorporation of the hydroxyethylene transition state isostere ψ[CH(OH)CH_2] has been much more effective. Placement of the Phe-Gly hydroxyethylene isostere in a consensus sequence for HIV-1 protease afforded the nanomolar inhibitors **19** and **20**. Similarly, insertion of the Leu-Val hydroxyethylene isostere into the *gag* sequence **2** provided inhibitor **21** with subnanomolar potency. The renin inhibitors **22** and **23** have served as lead structures for the design of potent hydroxyethylene and dihydroxyethylene-based HIV protease inhibitors, respectively. By analogy to inhibitors of pepsin, use of a phosphinate as a transition state mimic led to the subnanomar HIV protease inhibitor **24**. Interestingly, while Phe-Pro versions of the hydroxyethylene and phosphinate isosteres are drastically inferior to the corresponding Phe-Phe or Phe-Gly analogs (58,59), incorporation of the Phe-Pro hydroxyethylamine isostere ψ[CH(OH)CH_2N] into the p17/p24 cleavage sequence provided the potent inhibitors **25** and **26**.

Table 2. Peptide-based inhibitors of HIV protease.

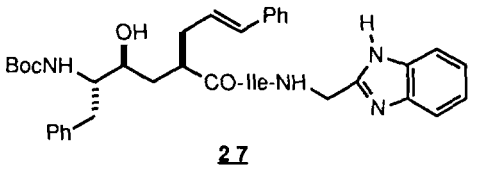
No.	Structure	K_i or IC_{50} (nM)	Ref.
11	Ac-Ser-Gln-Asn-Pheψ[CH_2-N]Pro-Val-Val-NH_2	14,000	(60)
12	H-Ser-Ala-Ala-Pheψ[CH_2-N]Pro-Val-Val-NH_2	1,900	(58)
13	H-Val-Ser-Gln-Asn-Pheψ[CH_2-N]Pro-Ile-Val-OH	3,520	(61,62)
14	Ac-Thr-Ile-Nleψ[CH_2-N]Nle-Gln-Arg-NH_2	789	(63)
15	Ac-Val-Val-Sta-Ala-Sta-OH	35	(64)
16	H-Val-Ser-Gln-Asn-Sta-Ile-Val-OH	3,690	(61)
17	Ac-Ser-Gln-Asn-AHPPA[a]-Val-Val-NH_2	39,000	(60)
18	H-Ser-Ala-Ala-AHPPA[a]-Val-Val-OMe	810	(58)
19	Cbz-Ala-Ala-Pheψ[CH(OH)CH_2]Gly-Val-Val-OMe	48	(65)
20	H-Ala-Ala-Pheψ[CH(OH)CH_2]Gly-Val-Val-OMe	18	(58,65)
21	H-Val-Ser-Gln-Asn-Leuψ[CH(OH)CH_2]Val-Ile-Val-OH	<1	(62,66)
22	Boc-Phe-Phe-Pheψ[CH(OH)CH_2]Phe-Leu-Phe-NH_2	1.0	(67)
23	Boc-Phe-His-Leuψ[CH(OH)CH(OH)]Val-Ile-Amp[b]	28	(68)
24	Boc-Val-Val-Pheψ[P(O)(OH)]Phe-Val-Val-NH_2	0.4	(59)
25	Ac-Ser-Leu-Asn-Pheψ[CH(OH)CH_2N]Pro-Ile-Val-OMe	0.24	(69,70)
26	Cbz-Asn-Pheψ[CH(OH)CH_2N]Pro-O-*t*-Bu	140	(71)

[a]AHPPA = 4(*S*)-amino-3(*S*)-hydroxy-5-phenylpentanoic acid; [b]Amp = 2-(aminomethyl)pyridine

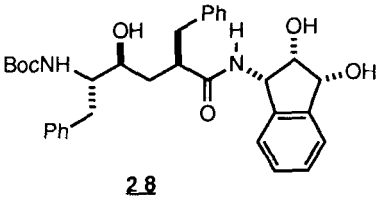
Systematic structure-activity studies on a number of the above lead compounds have provided inhibitors of unique structure and potency. Truncation of the amino terminus of **22** and variations at the P_1' - P_3' sites led to the exquisitely potent (IC_{50} = 0.03 nM) inhibitor **27** (67) as well as the further truncated structure **28** (IC_{50} = 0.1 nM) (72). Similarly, modification of **21** afforded **29** (K_i = 70 nM) (73), and optimization of **23** led to the subnanomolar inhibitor **30** (68). A systematic study of over 100 analogs of **26** provided the potent hydroxyethylamine based inhibitor **31** (IC_{50} < 0.4 nM) (71). Notably less peptide-like in their structure, these inhibitors are specific for HIV protease and effectively block the proteolytic processing of *gag* proteins in cell culture assays (*vide infra*). The subsite specificity for inhibitors of HIV protease closely resembles that for substrates. Thus, lipophilic, unbranched side chains (especially benzyl or cyclohexylmethyl) are favored in the symmetry-related P_1 and P_1' subsites while there is a clear preference for Ile, Val or Asn (or the truncated equivalent) at the P_2 and P_2' sites. Planar, aromatic end pieces, which may mimic the peptide backbone rather than the P_3 and P_3' side chains, are an additional common feature of many inhibitors.

A novel, structure-based approach to HIV protease inhibitors has recently been reported in which the symmetry characteristics of the inhibitor were designed to complement the C_2 symmetric active site of the enzyme. Conceptually, an axis of rotation through the tetrahedral intermediate **10** was defined, the P' region was deleted, and the remainder was subjected to a C_2 operation to generate symmetric core units (74,75). Placement of the axis of symmetry either through the carbonyl carbon or through the middle of the scissile bond provided the lead compounds **32** (IC_{50} = 3 nM) and diastereomeric diols **33** - **35**, respectively. The hydrated form of the corresponding quasi-symmetric difluoroketone **36** is also a potent inhibitor (IC_{50} = 0.1 nM) (76). Although still peptide-like in nature, these structures represent the first potent inhibitors of HIV protease to be

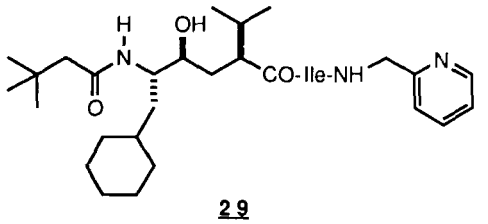
designed on the basis of both active site and substrate structure. In contrast to inhibitors based on hydroxyethylene or hydroxyethylamine isosteres, the inhibitory potencies of the symmetric inhibitors **33** (*R,R*; IC_{50} = 0.22 nM), **34** (*S,S*; IC_{50} = 0.38 nM) and **35** (*R,S*; IC_{50} = 0.22 nM) show little stereochemical dependence (58,67,69,71,74). Curiously, the optimal stereochemistry of the hydroxyl group actually differs between the two series of hydroxyethylamine inhibitors typified by **25** and **31** (69,71), an observation that has been rationalized by the absence of a P_3' residue in **32** (70) and by conformational differences in the P_1' site (77). Clearly, an exact definition of the modes of binding for different inhibitors will ultimately depend on X-ray crystallographic and NMR spectrometric methods.



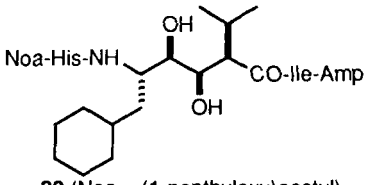
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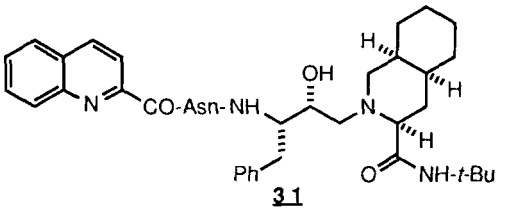
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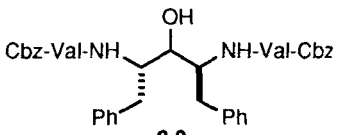
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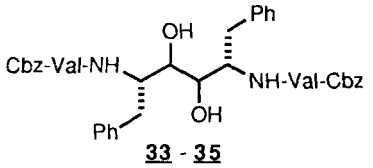
30 (Noa = (1-napthyloxy)acetyl)



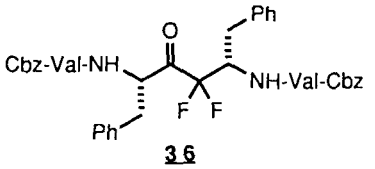
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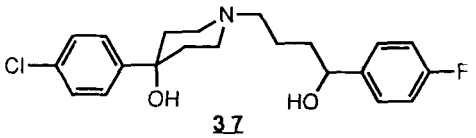
32



33 - **35**



36



37

The X-ray crystallographic structures of HIV-1 protease/inhibitor complexes with five different classes of inhibitors (**14**, **15**, **21**, **25** and **32**) have already been solved (25,57,70,75,78). The structure of the protease in each complex is similar to that of the unliganded dimer (21-23) with the notable exception of a flexible "flap" region. Sophisticated dynamics simulations appear to confirm a jaw-like action which would allow substrates and inhibitors access to the active site (79). As with other aspartic proteinases, inhibitors with secondary alcohol functionality at the scissile site are within hydrogen bonding distance of the two catalytically active aspartate residues. A comparison of the P' region of unsymmetric (**21**) and symmetric (**32**) inhibitors (Figure 2) reveals the same hydrogen-bonding and hydrophobic interactions within the active site despite the fact that the peptide residues in one are retroinverted in relation to the other. Indeed, the ability of unsymmetric

inhibitors to adopt a "symmetric" conformation, as evidenced by the electron density maps, is apparently the key to tight binding, since the overall two-fold symmetry of the enzyme is not strongly compromised in those complexes. The most remarkable feature of all of the protease/inhibitor complexes is the presence of a water molecule which bridges the P_2 and P_1' carbonyls of the inhibitor to the NH residues of Ile_{50} from each flap (Figure 2a). Since the various transition-state analogs are of different length, this bridging, ranging from eight atoms in **15** to ten atoms in **25**, results in significant kinking of the backbone of **25** and forces the proline ring into the P_1' pocket (70). This deviation from an extended backbone may account for the observation that Pro residues are unacceptable in the P_1' position of shorter hydroxyethylene and phosphinate-based inhibitors but favored in the longer hydroxyethylamines **25** and **26**. Indeed, the strain induced by bridging the residues of a substrate through a single water molecule may be a factor which allows retroviral proteases to cleave at the N-terminus of proline residues. The structures of **15** and **21** have in fact provided the basis for an elegant and detailed proposal for the catalytic mechanism of substrate cleavage by HIV protease (25).

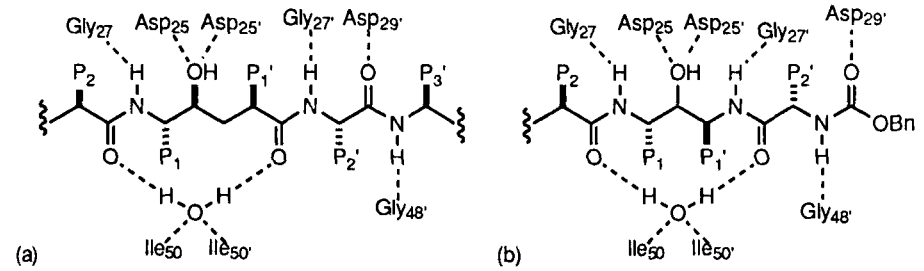


Figure 2. Partial schematic representation of the hydrogen-bonding interactions of inhibitors (a) **21** and (b) **32** with HIV-1 protease.

The availability of X-ray crystallographic structures of HIV protease/inhibitor complexes will undoubtedly encourage efforts toward the *de novo* design of non-peptide inhibitors which take advantage of specific hydrogen bonding and hydrophobic interactions with the active site. So far, a model study utilizing the X-ray structure of Rous sarcoma virus protease has been used used to support the placement of the axis of symmetry in the design of the symmetric inhibitor **32** (75). In addition, a computer-assisted search of the Cambridge Crystallographic Database based on the negative image of the active site of uncomplexed HIV-1 protease identified the antipsychotic agent haloperidol (**37**) (80). Although only a weak inhibitor of HIV protease (K_i ~ 100 μM), the novel structure of **37** may serve as a template for the design of other non-peptide structures. With the availability of coordinates for protease/inhibitor complexes, which differ significantly from the uncomplexed protease, additional novel, non-peptide inhibitors can be anticipated.

Antiviral Activity of Protease Inhibitors - The *in vitro* evaluation of any new antiviral agent is complicated by the wide range of tissue culture assays available and the need to carefully discriminate between cellular toxicity (TC_{50}) and antiviral activity (IC_{50}) (81). As summarized in Table

Table 3. *In vitro* anti-HIV activity of protease inhibitors.

No.	Virus Strain	Target Cell	Type of Infection	Antiviral Endpoint	IC_{50} (nM)	TC_{50} (μM)	Ref.
28	HIV-1_{3B}	H9	acute	% cells infected	100[a]	?	(72)
27	HIV-1_{3B}	H9	acute	% cells infected	12[a]	?	(67)
35	HIV-1_{3B}	H9	acute	p24 antigen	20	60	(74)
35	HIV-1_{3B}	MT4	acute	viral CPE	16	>100	(74)
31	HIV-1_{RF}	C8166	acute	p24 antigen	2	≥5	(71)
31	HIV-1_{GB8}	JM	acute	syncytia	2.5	≥5	(71)
31	HIV-1_{RF}	H9	chronic	infectivity	<10,000[b]	?	(71)
30	HIV-1_{LAV}	PBMC	acute	p24 and RT	<1,000	>10	(68)
30	HIV-1_{LAV}	CEMx174	chronic	viral CPE and RT	<1,000	?	(68)
19	HIV-1_{3B}	Molt 4	acute	RT, p24, syncytia	<4,200	?	(65)
29	HIV-1_{3B}	PBL	acute	p24, HIV RNA	<1,000	>10	(73)

[a]Concentration required to prevent virus spread beyond initially infected cells. [b]>3 log_{10} reduction at 10 μM.

3, a variety of target cells, virus strains, and endpoints have been used to evaluate the *in vitro* antiviral activity of protease inhibitors. While the independent demonstration of potent activity against HIV-1 in several systems is reassuring, the differences between these assays unfortunately preclude comparative judgments. Furthermore, direct comparisons of protease inhibitors with anti-retroviral agents acting at different targets may have limited value in attempting to predict relative therapeutic efficacy. Reverse transcriptase inhibitors, for example, act at an early stage in the viral life cycle, and are therefore quite effective against large viral inocula. Protease inhibitors, on the other hand, may have little effect prior to integration, and their subsequent ability to prevent the assembly of infectious progeny at a late stage in the viral life cycle could be overwhelmed if inordinate numbers of proviruses were produced in the primary infection. Assays designed to highlight the ability of protease inhibitors to limit the spread of infection in cell culture may therefore have the greatest clinical relevance.

Several measures of the extent of viral infection in a cell culture are available. The percentage of infected cells in a culture can be measured directly by fluorescent antibodies with specificity for viral proteins such as p24. With an initial multiplicity of infection of 1%, compounds **28** and **27** completely inhibited the spread of HIV-1_{3B} infection in H9 cells at concentrations of 100 and 12 nM, respectively. The total quantity of the viral p24 antigen produced by acutely infected H9 cells has also been used as an endpoint. In this assay, compound **35** had an IC_{50} = 20 nM. The same compound reduced the cytopathogenic effects (CPE) of HIV-1_{3B} in MT4 cells by 50% at 16 nM. One advantage of CPE based assays is that cytotoxicity cannot be confused with antiviral activity. Although the IC_{50}/TC_{50} ratio for compound **35** was greater than 6000, apparent antiviral activity at concentrations approaching cytotoxic levels should always be interpreted with caution, particularly when different endpoints are used to determine antiviral and cytotoxic effects (81). When p24 antigen production was used as an indirect measure of viral replication in C8166 cells acutely infected with HIV-1_{RF}, compound **31** had an IC_{50} of 2 nM. Syncytia formation in JM cells infected with HIV-1GB8 has also been used as an endpoint, and in this assay, compound **31** was 50% effective at 2.5 nM. In H9 cells chronically infected with HIV-1_{RF}, 10 μM **31** reduced the infectivity of the cell free supernatant more than a thousand-fold. As expected, 10 μM AZT had no effect on the production of infectious virus from chronically infected cells. At a concentration of 1 μM, inhibitor **30** was reported to completely block the spread of HIV-1_{LAV} infection in primary peripheral blood mononuclear cells, as measured by supernatant reverse transcriptase activity and p24 antigen level. Inhibitor **30** also prevented cell death and the appearance of reverse transcriptase activity in chronically infected CEMx174 cells for at least 4 weeks. In the absence of drug, all cells were dead within eight days. At a concentration of 4.2-25 μM, the hydroxyethylene isostere **19** virtually eliminated syncytia formation, particle associated reverse transcriptase activity, and supernatant p24 antigen in Molt 4 cells acutely infected with HIV-1_{3B}. Another hydroxyethylene isostere, **29**, supressed the production of p24 antigen and HIV RNA in acutely infected peripheral blood lymphocytes by at least 50% at concentrations between 0.1-1 μM.

In summary, the biological consequences of chemical inhibition of the HIV protease appear to be identical to those caused by disabling mutations of the catalytic site. Several laboratories have demonstrated the accumulation of unprocessed viral polyproteins in the presence of protease inhibitors, and one group has found that protease inhibition in an HIV-1 like particle by **30** (K_i < 1 nM) was essentially irreversible (68). Electron micrographs have revealed the corollary production of virions with immature morphology, and incubation of these particles with susceptible target cells has confirmed their lack of infectivity.

The selection of virus strains and target cells for the evaluation of protease inhibitors deserves additional comment. While early efforts have tended to focus on establishing antiviral activity against a few lab strains of HIV, clinical isolates of HIV can vary significantly in genetic makeup, pathogenicity, and cellular tropism; in some cases these factors appear to correlate with the progression of AIDS (82,83). Central nervous sytem isolates, for instance, replicate efficiently in peripheral blood macrophages but are relatively incapable of infecting T-cells (84). Only minor differences in nucleotide sequence gave rise to HIV1-NDK, an African clinical isolate found to be 10,000 times more cytopathic to MT4 cells than a prototypical HIV strain (85). In light of this marked heterogeneity, the preclinical evaluation of protease inhibitors should ideally involve a panel of primary target cells and clinical isolates, including AZT resistant strains.

Future Prospects - As for any targeted antimicrobial agent, the possibility of clinical resistance to protease inhibitors, as a class or as individual agents, must be recognized. Clinical resistance to AZT was not preceded by the selection of resistant mutants *in vitro*, so the conspicuous lack of reports regarding *in vitro* resistance to protease inhibitors provides little comfort. It can be noted however that single, non-conservative mutations in the protease binding cleft consistently produce

a protease negative phenotype. Overall, 40% of the protease's 99 amino acids are sensitive to mutation (32,86), and about 50% of the amino acids in the HIV-1 and HIV-2 proteases are absolutely identical (66). Modeling studies predict that of the amino acids which would come into contact with an inhibitor, only three symmetry related pairs differ in the two enzymes, and these differences merely involve the interchange of valine and isoleucine. This high degree of conservation presumably reflects the stringency of specific cleavage at eight distinct sites, and the low probability of simultaneous, compensating mutations in the protease and its substrates (32,33). Nonetheless, the subtle structural disimilarities between HIV-1 and HIV-2 are sufficient to generate more than 100-fold differences in the binding affinity of certain inhibitors (66). Heterologous *pol* genes, in which the HIV-1 and HIV-2 protease domains are interchanged, do in fact release reverse transcriptase and integrase, albeit less efficiently than the homologous systems (87). Other studies with model substrates have shown that HIV-2 protease is capable of processing many of the HIV-1 cleavage sequences (88-90). Thus, the emergence of resistance to a specific protease inhibitor in a given individual is a real, even if unlikely, possibility.

Virtually all of the potent inhibitors of the HIV protease discussed in this review share certain physicochemical characteristics: a high molecular weight, low solubility, high lipophilicity, and significant peptide character. These attributes reflect the ancestry of renin inhibitors as well as the nature of the HIV protease binding cleft. Historically, peptide based leads have presented significant obstacles to drug development (91). Indeed, the low oral bioavailability and rapid biliary excretion exhibited by most renin inhibitors have foreshadowed similar difficulties with inhibitors of the HIV protease (9). However, recent work in the renin area has shown that systematic structural modifications can lead to compounds with acceptable pharmacodynamic properties (92). Efforts to design inhibitors based on protein structure rather than substrate sequence may, in time, offer another avenue to drug candidates. Random screening with high throughput assays might also yield useful, non-peptide leads.

Given the life-threatening nature of AIDS, the delivery of protease inhibitors *via* non-oral routes, such as intravenous or subcutaneous injection, may also have a role in therapy. The absence of proven animal models for AIDS could mean that the first evidence of *in vivo* efficacy for such a compound might emerge in human subjects (93). If a therapeutic benefit were observed, combination therapy with AZT or other reverse transcriptase inhibitors would certainly be considered. So far, however, synergistic, *in vitro*, anti-retroviral activity between protease and reverse transcriptase inhibitors has not been reported.

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Chapter 16. Radiosensitizers

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Introduction - Radiation therapy continues to be an important treatment modality for many forms of human cancer. Although this therapeutic approach is effective in many cases, normal tissue toxicity limits the total dose of radiation a patient can ultimately receive. Also, many tumors contain areas of diminished oxygen concentration which make them more resistant to the lethal effects of radiation. Advances in instrumentation and technology have provided radiotherapists with the means of targeting x-rays more precisely and eliminating some of the normal tissue damage, but not enough to eliminate the problem totally. Since the number of patients diagnosed with cancer will exceed one million in 1990, and 50-60% of these patients will undergo some type of radiation treatment, methods which increase the effectiveness of clinical radiotherapy are potentially very useful (1). Fortunately, physiological differences that exist between tumor cells and normal tissues have been exploited to develop a number of clinical candidates. However, most of these compounds have failed due to a lack of potency or as a result of direct toxicity of the drug at therapeutic doses (2). These studies, although clinically unsuccessful, have provided a great deal of information which has been useful in the design and development of new approaches and agents (3).

This chapter will review progress toward enhancing the effectiveness of radiotherapy. It will cover the use of chemical agents which, through a variety of mechanisms, selectively sensitize tumors to the lethal effects of x-rays. Since this is the first time that radiosensitizers have been covered in this forum, an effort has been made not only to include the most recent literature, but where appropriate, to include previous references which illustrate a novel or interesting approach. A number of recent reviews address in more detail the topics highlighted above (4), the rationale for the development of such compounds (5), potential mechanisms and points of intervention (6), as well as the clinical aspects of radiobiology (7).

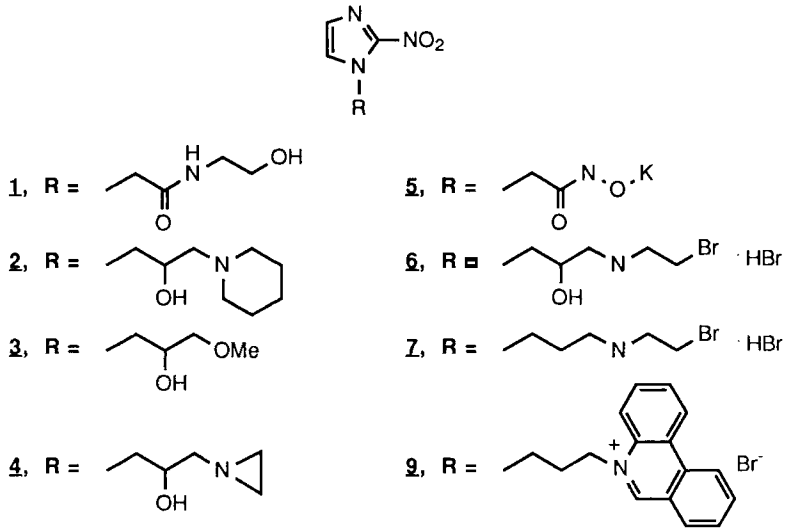
A variety of methods have been evaluated in attempts to overcome the radioresistance of tumor cells. The most widely studied approach includes the use of oxygen mimetics, which are compounds with specific physical properties (reduction potential, pKa and log P) that are able to penetrate into tumors and cause DNA damage by a mechanism similar to that of oxygen. Also under evaluation are modifiers of oxygen utilization which increase the oxygen concentration in tumors or inhibit its metabolic use, and repair inhibitors, which are compounds that interfere with the processes involved in the repair of radiation-induced DNA damage.

Oxygen Mimetics - A large number of nitroheterocycles have been evaluated as oxygen mimetics, although some work has been done in an attempt to find non-nitro compounds acting via the same mechanism (8-10). However, many of the non-nitro compounds evaluated were quickly metabolized and therefore not active in vivo. Of the nitroheterocycles studied, the 2-nitroimidazoles exhibited the best overall properties, which could not be explained solely on the basis of their reduction potentials (11). Many of the compounds evaluated clinically are of this class with etanidazole (SR 2508, **1**) and pimonidazole (Ro-03-8799, **2**) still under active evaluation (4-7). A substantial increase in radiosensitizing activity, both in vitro and in vivo, was seen when an alkylating moiety was combined with the 2-nitroimidazole (12). An aziridine group was substituted for the methoxy group of misonidazole (**3**) to produce RSU 1069 (**4**). This was the first "DNA-targeted" radiosensitizer to be studied clinically; however, the reactivity of the aziridine led to unmanageable emesis and the compound was dropped (13). In an effort to decrease this side effect, yet retain

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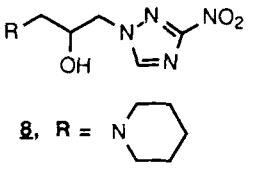
the radiosensitizing activity, the aziridine was replaced by substituted aziridines (14), cycloalkyl-aziridines (15,16), acetohydroxamic acids (17), chloroethylnitrosoureas (18) and ß-haloethylamines (19,20). KIH-802 (**5**), the most potent of the acetohydroxamic acid analogs, displayed significant radiosensitizing activity in vivo in SCCVII tumors (21). Pharmacokinetic studies revealed that levels of **5** in neuronal tissue were quite low suggesting that the compound would have a minimal tendency to cause neurotoxicity. Further studies on this compound are progressing as is additional work on other hydroxamic derivatives that can stabilize radicals in a similar manner (17). The use of ß-haloethylamines resulted in two compounds, RB 6145 (**6**) and the corresponding desoxy derivative, PD 130908 (**7**). These aziridine prodrugs were equal in activity to **4**, but less toxic (22). Studies to select a potential clinical candidate have been initiated (23).

Combining reactive side chains with aza-2-nitroimidazole (3-nitro-1,2,4-triazole) did not result in an increase in radiosensitizng potency as was observed for the 2-nitroimidazoles. Of those anaolgs synthesized the best activity was displayed by compound **8**, which contained a piperidine moiety (24,25).

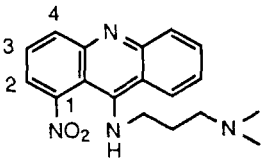


A rationale to increase the selectivity of oxygen mimetics by targeting them to the DNA of hypoxic cells led to the design of compounds combining alkylating moieties with 2-nitroimidazoles. DNA intercalating groups have also been employed in this fashion. For example, linking a phenanthridine to a nitroimidazole resulted in (NLP-1, **9**) that bound to DNA as measured by ethidium bromide displacement, was selectively cytotoxic to hypoxic cells (8-fold) and produced radiosensitization in vitro (26). The radiosensitization observed with this compound was somewhat less than expected and studies are underway to determine if this resulted from reduced drug levels under the test conditions or to a lack of effective targeting. Studies on a series of nitroacridine positional isomers were also reported (27). Nitracrine (**10**) has been shown to be a "DNA-targeted", hypoxia-selective radiosensitizer (28). Studies on its isomers have demonstrated differences in reduction potential, hypoxic cell cytotoxicity, aerobic toxicity and hydrolytic and reductive stability. The 2,- 3,- and 4-positional isomers showed similar sensitization, but were less potent than the parent compound, (**10**). These differences have been attributed to the fact that these compounds have higher DNA association constants than **10**, indicating that kinetically stable binding of the compounds to DNA prevents the rapid diffusion required to access target molecules.

Another form of DNA-targeted drug design was illustrated by the use of antibodies directed against tumor cell surface antigens. Earlier studies demonstrated the feasibility of applying this concept (29,30). Recent work has focused on a series of 4-nitro-5-sulfonatoimidazoles as potent



8, R = N

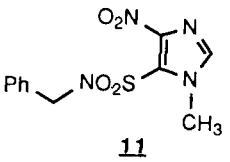


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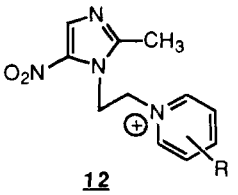
agents for conjugation to an antibody targeted to colorectal carcinoma, which, in clinical situations, has frequently been less responsive to radiotherapy than other tumor types (31). The compounds were evaluated in vitro against a human colon adenocarcinoma cell line (SW1116), and several members of this series were found to be 40-300 times more effective radiosensitizers than misonidazole. The activity of these compounds was not correlated to reduction potential, glutathione reactivity, capacity factor, or cytotoxicity. The benzyl derivative (11) was found to have the greatest sensitizing efficiency and is presently undergoing stabilization studies for possible antibody attachment (31).

A study was conducted whereby the calculated energy of the lowest unoccupied molecular orbital was shown to correlate to the measured reduction potentials and to the in vitro radiosensitizing activity for a series of 5-nitroimidazoles (32,33). Additional studies were performed to prove that the activity seen with these compounds (12) was indeed related to their reduction potentials and not to the inhibition of DNA synthesis or other related processes (34,35).

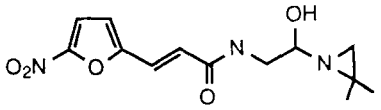
In an attempt to increase the radiosensitizing activity by raising the reduction potential, a series of 5-nitrofurans (13) containing a variety of side chains was synthesized (36). Although these compounds were potent radiosensitizers in vitro, they were, in general, more cytotoxic to oxic cells and were inactive in vivo. This lack of in vivo activity was postulated to result from metabolism, poor distribution and/or other biological interactions that removed the compounds before they could reach the tumors.



11



12

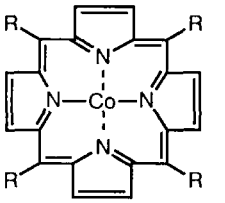


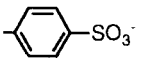
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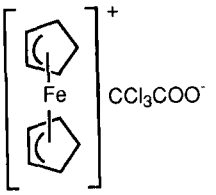
Antitumor platinum complexes, such as cis-platinum have also been shown to be radiosensitizers. They impart their radiosensitizing activity via an oxygen-mimetic mechanism similar to that of nitroheterocycles, whereby reduction of the metal occurs resulting in subsequent fixation of the radiation-induced DNA damage (37). They may also sensitize through a number of related mechanisms (38). Platinum complexes have been combined recently with a variety of nitroheterocycles to provide yet another means of delivering a potential sensitizer to its intended target (39). The compounds reported had moderate activity as radiosensitizers both in vitro and in vivo. They bind to DNA, as measured by the inhibition of restriction enzyme cleavage and their activity was potentiated by the concurrent use of vasodilators.

Other metal complexes have also been used as radiosensitizers, specifically in conjunction with porphyrins (40,41). Porphyrins are known to accumulate selectively in some tumors and have been used extensively as photosensitizers. Metal/porphyrin complexes designed to take advantage of this selectivity were prepared and evaluated. Six different metals (Co, Cu, Zn, Fe, Pd, Mn) in combination with substituted porphyrins were investigated. Of those tested, the cobalt complex of tetrakis(4-sulfonatophenyl)porphyrin (**14**) had in vitro activity superior to that of other metals. Additionally, selectivity for hypoxic cells over oxic cells was observed in the radiosensitization studies.

A group of non-organic compounds with similar redox properties to the nitroimidazoles was tested as radiosensitizers. Two ferricinium salts, trichloroacetate and hexafluorophosphate (**15**), as well as an alkyl-substituted analog, t-pentyl ferricinium hexafluorophosphate, were prepared (42). Only the first two compounds were tested as radiosensitizers since the lipophilic nature of the alkyl analog resulted in considerable toxicity due to increased cellular uptake. Differences in toxicity between oxic and hypoxic cells were observed when serum proteins were added, with oxic cells becoming less susceptible to the compounds. Moderate radiosensitizing activity was observed and this result, combined with the differential toxicity data, suggested an area for further study.



14, R = 

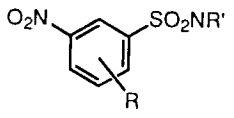


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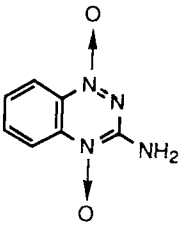
In an attempt to eliminate the liabilities of nitroimidazoles, other nitroaromatic compounds have been evaluated for radiosensitizing activity. These include nitrobenzofurazans (43), 1-alkyl-3-nitropyrrolopyridines (44), 3-nitro-4-quinolones (45) and 3-nitrobenzenesulfonamides (46). This last group of compounds (**16-18**) has been reported extensively in the patent literature as radiosensitizers (46-49).

SR 4233 (**19**), a potent and selective hypoxic cell cytotoxin, that caused extensive cell death in the presence of radiation, has been shown recently to be effective in a clinically relevant fractionated radiation protocol (50,51). In vivo studies, using four different tumors varying in their hypoxic fraction, compared **19** to **1**. The data indicate that **19** resulted in a significant enhancement of cell killing over radiation alone. In two of the tumors studied, no effect was observed with **1**, whereas **19** was effective in all of the tumor systems tested. Since the compound was active when given either prior to or after radiation, it is thought that direct killing of hypoxic cells rather than radiosensitization was responsible for its effect. Mice were able to tolerate administration

of this compound for up to six weeks, and toxicity studies indicated no adverse effects on normal tissues. The preclinical data suggests that this compound has considerable promise as an adjunct to clinical radiotherapy (51).



16, R = 4-$CR_3R_4NO_2$
17, R = 5-NO_2
18, R = 6-CNR_1R_2 (‖ O)



19

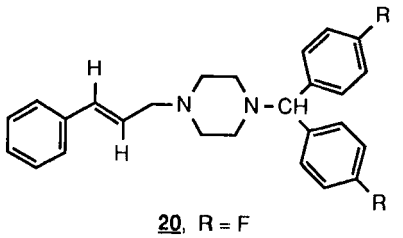
Oxygen Modifiers - Sensitization of tumors can also be achieved by increasing the concentration of oxygen in tumors. The first evaluation of this approach involved the use of hyperbaric oxygen. Clinical trials were conducted, but failed to indicate an enhancement over radiation alone (52). Although the concentration of oxygen in blood was increased, it was unclear whether this resulted in a corresponding increase of oxygen concentration in the tumors. Perfluorochemicals have been utilized to increase oxygen- carrying capability of the blood and have been shown to increase the radiosensitivity of mouse tumors. Fluosol-DA● (20%) is currently in Phase 3 clinical trials as a radiosensitizer. Recent animal studies have shown that Fluosol-DA in combination with carbogen breathing (95% oxygen/ 5% carbon dioxide) increases the intratumoral oxygen levels, thereby decreasing the hypoxic fraction (53). Significant radiosensitization of KHT tumors, using a fractionated protocol, was obtained. Additional studies using the laser Doppler technique demonstrated that tumor blood flow was increased with no substantial increase in skin blood flow. Some toxicities have been observed with Fluosol-DA and studies examining other more stable, less toxic perfluorochemical emulsions are underway. For example, more concentrated emulsions based on bis(perfluorohexyl)ethylene (F66E) and bromoperfluorooctane (PFOB) have been studied in animals (54). These perfluorochemicals carry more oxygen than Fluosol, which contains a mixture of perfluorodecalin and perfluorotripropylamine, and are obtained by chemical syntheses providing a highly purified fluorocarbon. Of interest is the fact that PFOB has a much shorter retention time in organs than other perfluorochemicals (4 days compared to 65 days for F-tripropylamine and 400 days for F66E). These new emulsions had only weak activity as radiosensitizers, but caused no increase in lung metastases and the effect of PFOB at five times the dose on the liver, spleen, lung and overall body weight was no greater than that seen with Fluosol. The decreased toxicity of these compounds warrants further studies in comparative trials with Fluosol.

A more concentrated emulsion, Therox ●, containing bis-perfluorobutyl ethylene (F44E), has been evaluated in vivo to determine the effect of changes in the injection volume and/or concentration of the perfluorochemical on radiation-induced growth-delay of Lewis lung carcinoma (55). The emulsion alone had no effect, but in combination with carbogen breathing, concentrations of the emulsion varying from 48% to 12% (v/v) and injection volumes from 0.4 to 0.1 ml resulted in a substantial increase in growth-delay. The studies indicated that the most important parameter was the injection volume with a dose of 4 mg/kg in a volume of 0.2 ml showing the best activity. An equivalent dose of Fluosol-DA would have to be given in a volume of 0.5 ml resulting in significant stress to the animal.

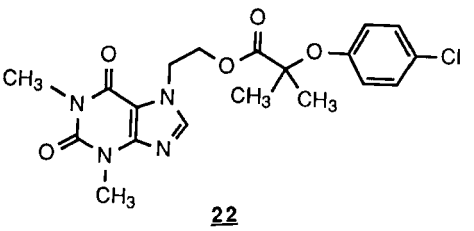
Increasing the blood flow to tumors represents another approach to increasing their sensitivity to radiation. The calcium antagonists verapamil, nifedipine and flunarizine, which each modify host blood flow by a different mechanism, were evaluated for their ability to decrease hypoxia and sensitize SCCVII/St tumors to radiation (56). At higher doses verapamil and nifedipine increased cell survival after radiation and increased the hypoxic fraction of the tumor as determined by Hoescht 33342 staining. This radioprotective effect was attributed to the "steal phenomenon", whereby decreases in blood pressure result in a decrease in blood flow to the tumors. At lower doses, the opposite effect was observed. A 30% reduction in the hypoxic fraction was seen, but very little radiosensitization accompanied this effect. Flunarizine (**20**), at all doses, produced significant sensitization of the tumor although there was only a 20% reduction in tumor hypoxia. The discrepancy between the reduction in tumor hypoxia and the radiosensitization seen with these compounds was attributed to the fact that **20** can prevent the occurrence of hypoxia-induced rigidification of red blood cells whereas the other compounds cannot. A closely related, but less potent analog, cinarizine (**21**) was also examined (57). Studies using the Rif-1 tumor, which contains a smaller hypoxic fraction than the SCCVII tumor used previously, showed that **21** was a much weaker sensitizer than **20** in the SCCVII model and was inactive in the Rif-1 tumor. Compound **20**, which was active in both systems, was assessed for normal tissue toxicity and was found to have no effect on the total white blood cell count. Additional studies using fractionated radiation regimens have been initiated.

Indomethacin, has also been evaluated as a radiosensitizer. This compound is a calcium antagonist and reacts with hydroxy radicals to produce a species toxic to cells (58). It was also shown to sensitize fibrosarcomas to radiation (59). Growth delays of nine days for single radiation doses and six days for fractionated doses, with 2/10 cures in each study, were obtained. The exact mechanism for the activity was not presented, but may result from a combination of all the aforementioned effects. This drug is currently used clinically as an antiinflammatory agent, therefore data regarding metabolism, toxicity and pharmacokinetics are available and should facilitate additional radiosensitizer studies (60).

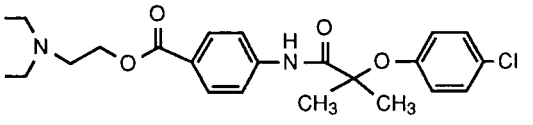
Some chlorophenoxyacetic acid derivatives, which have clinical utility as lipid lowering agents, have also been found to reduce the affinity of hemoglobin for oxygen (61). This reduced binding to hemoglobin increases the availability of oxygen to hypoxic regions of tumors. The hypoxic



20, R = F
21, R = H



22



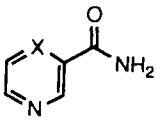
23

fraction of SCCVII tumors was reduced by 30-40 fold by treatment with clofibrate resulting in sensitization of these tumors to radiation. Two less toxic analogs, ML1024 (**22**) and ML1037 (**23**), had a similar effect on hemoglobin affinity, but displayed very little radiosensitization. Apparently, changes in the hemoglobin affinity, and the resulting increase in plasma oxygen concentration, is not the sole mechanism by which clofibrate sensitizes tumors to radiation.

*Nicotinamide (**24**) and pyrazinamide (**25**) have also been shown to produce small,* but significant, changes in tumor blood flow (62-65). Sensitization studies with a variety of tumor types confirm that these changes in blood flow do result in the sensitization of tumors to the lethal effects of radiation. Only a slight effect was seen in normal tissues as assessed by examining mouse crypt cells, indicating a potential therapeutic advantage. Camphor also sensitized tumors to radiation, but by a more general decrease in oxygen consumption by normal cells (66). This made more oxygen available for penetration into hypoxic areas. The compound was relatively non-toxic and resulted in significant sensitization of tumors (increased growth-delay) with a number of cures at the higher doses.

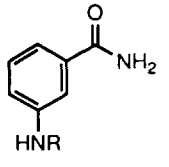
Repair Inhibition - The use of agents which inhibit the repair of radiation-induced DNA damage in the tumor is more complex and less clearly defined than previously discussed areas. A number of enzymatic processes are thought to be involved in DNA repair and which of these are critical has been widely disputed (67). Agents that are classified as repair inhibitors can act by a variety of mechanisms, but usually have in common the fact that they inhibit cellular repair processes after radiation (68).

One enzyme believed to regulate or modify the repair of radiation-induced DNA damage is poly(ADP-ribose) polymerase (69). An inhibitor of this enzyme, 3-aminobenzamide (**26**), has been shown to sensitize cells to radiation and inhibit cellular repair processes (61). Originally, it was thought that **24** fell into this class of radiosensitizers and that its activity was due to inhibition of this enzyme. However, more recent evidence suggests that **24** sensitizes tumor cells to radiation solely by virtue of its effect on tumor blood flow (65). Newer compounds postulated to act as repair inhibitors all are structurally related to the benzamides. A series of rigid benzamide analogs, the dihydroisoquinolinones, were shown to be more potent inhibitors of the enzyme than 3-aminobenzamide, *and exhibited significant post-irradiation sensitization (70-73). One member of* this series, PD 128763 (**27**), was also shown to inhibit cellular repair processes, repair of radiation-induced DNA strand breaks and was active in vivo as a radiosensitizer (74-77). A correlation was seen between inhibition of the enzyme, poly(ADP-ribose)polymerase and inhibition of cellular repair. Additional studies are in progress both to optimize the in vivo activity and to elucidate further their mechanism of action. A series of 8-aminocarsalam derivatives (**28**) have been described in a patent as inhibitors of this enzyme and as radiosensitizers (78).

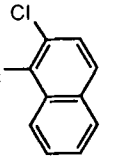


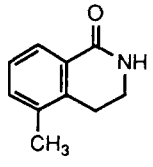
24, X = C

25, X = N



26, R = H

29, R = 

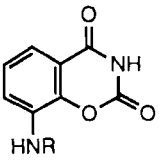


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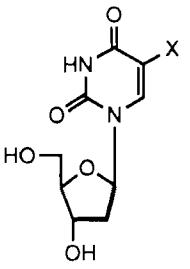
Several naphthylsulfonamides were shown to inhibit the repair of radiation-induced DNA double strand breaks (79). The most active member of the series, which is a 3-substituted benzamide analog (**29**), was active in vivo in EMT6 tumors (400 mg/kg, single dose of radiation) and exhibited a 16 day growth delay over radiation alone in SCCVII tumors.

Ouabain, a cardiac glycoside and potent inhibitor of the sodium-potassium pump, has been shown to radiosensitize tumor cells (A549 adenocarcinoma) selectively over normal cells (CCL-210 lung fibroblast) (80). Evidence for this compound acting as a repair inhibitor stems from the fact that the compound is active post-irradiation. Studies on this promising lead aimed at determining the mechanism of sensitization as well as the reason for the tumor selectivity were inconclusive and are continuing. The effects on potassium retention and protein synthesis were evaluated but found not to contribute to the selective radiosensitization of tumor cells.

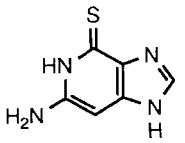
Miscellaneous - A clinical re-evaluation of the use of halogenated pyrimidines (**30**) as radiosensitizers was undertaken (81). Previous clinical studies were hampered by normal tissue toxicity and difficulties with drug administration. To be effective, these compounds require incorporation into DNA in place of thymidine, and therefore many days of continuous infusion are required to ensure sufficient levels in the tumors. To support ongoing clinical trials, studies examining the levels of incorporation, methods for modifying these levels, and the effect of various doses of radiation used have been initiated.



28



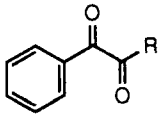
30



31

6-Thioguanine (6-TG, **31**) has been used clinically as an antileukemic agent for over 30 years. Studies aimed at determining whether this compound could sensitize mouse tumors to radiation have been carried out (82). At concentrations readily achievable in humans, **31** sensitized the Meth-A and Rif tumors to radiation in single dose fractionated protocols. In one study, activity was seen with **31** and radiation, whereas no activity was seen with radiation alone. Also, the compound produced no normal tissue damage over that seen with radiation alone. Although the mechanism of sensitization remains elusive, the data suggest that clinical studies with this compound should be pursued.

Five different glyoxals, were evaluated as radiosensitizers in vitro using TC-SV40 cells (83). The phenyl glyoxals (**32**,**33**) showed moderate radiosensitization of hypoxic cells and none of the compounds sensitized aerobic cells. Their effect on cellular levels of nonprotein sulfhydryls was measured, but it was concluded that their weak ability to deplete thiols did not account for their slight radiosensitizing effect.



32, R = H
33, R = OH

Conclusions - The search for new radiosensitizers has focused on obtaining more selective, less toxic compounds. More recently, approaches have investigated novel means of targeting compounds to DNA, modifying oxidative processes, and inhibiting the repair of radiation-induced DNA damage. Additional mechanistic studies in conjunction with carefully chosen clinical protocols will ultimately prove the utility of these compounds.

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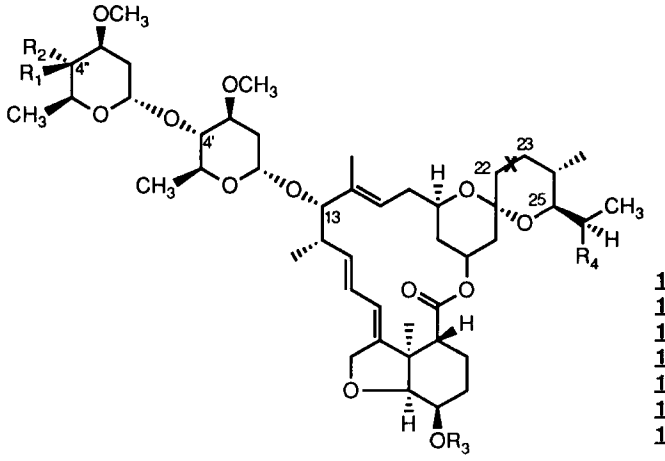
Chapter 17. Antiparasitic Agents

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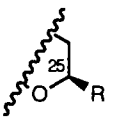
ANTHELMINTIC AND ECTOPARASITICIDAL AGENTS

Introduction - Chemotherapy for endo- and ectoparasitic infections has been dominated in recent years by the use of the avermectins and the closely related milbemycins. These compounds have found widespread use in veterinary health and limited utility in human health . They are also being used in crop protection as miticides and insecticidal compounds are now also in development (for recent reviews see references 1-4). Due to the efficacy and breadth of spectrum of these compounds, the majority of newly described anthelmintic agents have been based on synthetic, semi-synthetic and microbiological derivatives of the avermectins and this is reflected in the topics reviewed in this chapter.

Avermectins - A series of novel avermectins, 4"-amino-4"-deoxy avermectins, has been identified which has excellent insecticidal activity as assessed against the beet armyworm *Spodoptera*



(1-10)



(11-17)

11 $CH(CH_3)CH(CH_3)_2$
12 $C(CH_3)CHCH_3$
13 cyclohexyl
14 cyclohexanonyl
15 cyclohexenyl
16 2- thienyl
17 2- furanyl

	R_1	R_2	R_3	R_4	C_{22}-X-C_{23}
1	H_2N	H	H	> 80% C_2H_5 < 20% CH_3	-CH=CH-
2	H_3CHN	H	H	> 80% C_2H_5 < 20% CH_3	-CH=CH-
3	H	OH	CH_3	C_3H_7	-CH=CH-
4	H	OH	H	C_3H_7	-CH=CH-
5	H	OH	CH_3	C_3H_7	-CH_2CHOH-
6	H	OH	H	C_3H_7	-CH_2CHOH-
7	H	OH	CH_3	C_4H_9	-CH=CH-
8	H	OH	H	C_4H_9	-CH=CH-
9	H	OH	CH_3	C_4H_9	-CH_2CHOH-
10	H	OH	H	C_4H_9	-CH_2CHOH-

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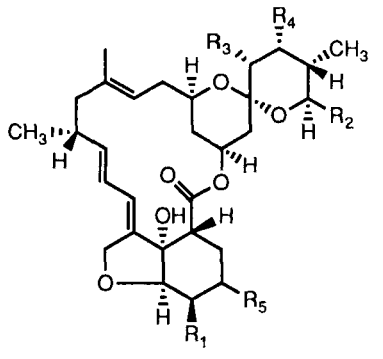
exigua, and other lepidopteran larvae (5). The most effective of these compounds (**1**,**2**) are 1,500-fold more potent than avermectin B_1 (abamectin).

New avermectin homologs were produced by *S. avermitilis* when externally supplied with sodium 2-methylpentanoate and sodium 2-methylhexanoate. The homologs carry 2-pentyl and 2-hexyl groups, respectively, at C-25 of the aglycone moiety as opposed to the 2-butyl group of "a" components and the isopropyl group of "b" components of natural avermectins. The new homologs designated as avermectin "c" and "d" components (**3**-**10**), possess potent anthelmintic and insecticidal activity (6).

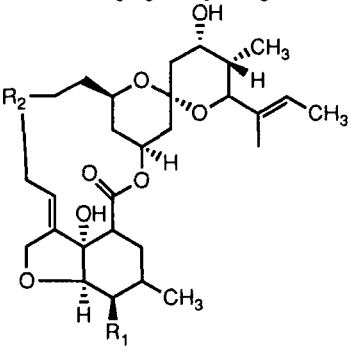
A number of novel 25-substituted avermectins have been prepared microbiologically by feeding carboxylic acids or their precursors to a mutant strain of *S. avermitilis*. The mutant lacks the ability to form isobutyric and S-2-methylbutyric acids from their 2-oxo acid precursors and is consequently unable to produce natural avermectins. A large number of analogs (including acyclic, unsaturated, cycloalkyl, heterocyclic and aromatic substituents, **11**-**17**) were identified and found to have potent anthelmintic activity (7,8).

A series of 13-substituted avermectin aglycons was synthesized using 5-O-(*tert*-butyldimethylsilyl)-22,23-dihydroavermectin B_{1a} aglycon as a starting material (9). Anthelmintic and insecticidal activities of these compounds were evaluated and it was determined that the lipophilic 13-substituents such as halogen and methoxy (**18**,**19**) retain high biological activity while the more polar (**20**,**21**) hydroxy and amino substituents had reduced biological activity.

Milbemycins - A new series of milbemycin-like macrolides (**22**-**25**) was isolated from *Streptomyces cyaneogriseus* containing an unsaturated side chain extending from C-25. The polyketide precursor of these compounds is composed of 14 acyl units as opposed to the precursors of milbemycins and avermectins which contain only 13 acyl units (10). The main component of this broth (**22**, nemadectin) is highly efficacious against natural infections of the major canine gastrointestinal helminths. At a single oral dose of 0.2-0.4 mg/kg body weight the liquid



(**22**-**25**, **29**-**34**)



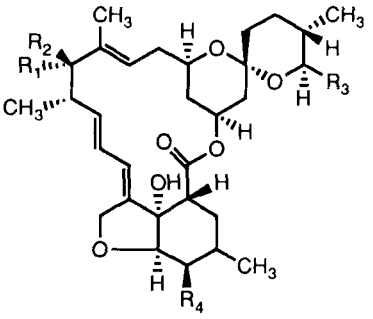
(**26**-**28**)

	R_1	R_2	R_3	R_4	R_5
22	OH	$C(CH_3)CHCH(CH_3)_2$	H	OH	CH_3
23	OH	$C(CH_3)CHCH_3$	H	OH	CH3
24	OCH_3	$C(CH_3)CHCH_3$	H	OH	CH3
25	OCH_3	$C(CH_3)CHCH(CH_3)_2$	H	OH	CH_3
26	OCH_3	$OCO\ CH_2CO_2$			
27	OH	$OCOCH_2CO_2$			
28	OH	O			
29	OH	$C(CH_3)CHCH_3$	H	H	CH_3
30	OCH_3	$C(CH_3)CHCH_3$	OH	H	CH_3
31	OCH_3	$C(CH_3)CHCH_3$	OH	H	$OCOC(CH_3)CHCH_3$
32	OH	$C(CH_3)CHCH_3$	OH	H	CH_3
33	=NOH	C_2H_5	H	H	CH_3
34	=NOH	CH_3	H	H	CH_3

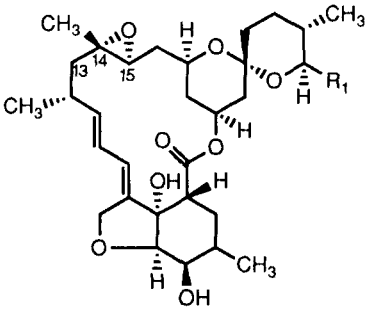
formulation was 100% effective in eliminating natural infections of *Toxocara canis*, *Toxascaris leonina*, *Ancylostoma caninum* and *Uncinaria stenocephala*; however, control of *Trichuris vulpis* infection required dosages of 0.6-0.8 mg/kg (11). The anthelmintic activity of **22** in experimentally infected sheep demonstrated a weakness against *C. oncophora* (12). Nemadectin was also evaluated as an acaricide, and a single injection of 3 mg/kg eliminated *Psoroptes ovis* for 35 days (13). Nemadectin has been modified to provide the semi-synthetic analogs (**26-28**) (14). The biological activity of this series of compounds has not yet been reported.

A family of four milbemycin metabolites produced by a *Streptomyces* (strain E225) was identified (**29-32**) containing an unsaturated C-25 side chain and either unsubstituted at C-22 and C-23 or possessing a single C-22 hydroxyl group (15). In naturally infected sheep, **29** removed all major nematode species at a dose of 0.4 mg/kg, whereas **32** at 0.8 mg/kg cleared most species, *Cooperia* being the most refractory (16).

Milbemycin oxime analogs constitute a group of semi-synthetic analogs of antibiotics originally isolated from the fermentation broth of *Streptomyces hygroscopicus aureolacrimosus* and identified as acaricidal as well as anthelmintic agents (17, 18). Dogs were dosed with 500 µg/kg of a mixture of the 5-oxime derivatives of milbemycin A4 (**33**) and milbemycin A3 (**34**) (80% and 20%, respectively) . This combination was efficacious for the precardiac and microfilarial stages of *D. immitis* and adult *T. canis*. These compounds were also effective in the treatment of adult *A. caninum*, but not *U. stenocephala* (19, 20). The diastereoselective hydroxylation of milbemycin A3, A4 and D at the 13-ß position using cultures of *S. violascens* (21) produced the corresponding 14,15-epoxides (**35-37**) which may serve as useful intermediates for the synthesis of 13ß-alkoxy, alkylthio, acyloxy, carbamoyloxy and alkyl derivatives. The synthesis of several configurationally defined hydroxymilbemycin derivatives was described (22), including a functionalization of milbemycin at position 13. Several allylic alcohol isomers were synthesized including 13ß-hydroxymilbemycin D (**38**) and the 5-O-protected 13-epimer (**39**); this represents a conversion of the milbemycin to the avermectin series.



(**18-21**, **38**,**39**)



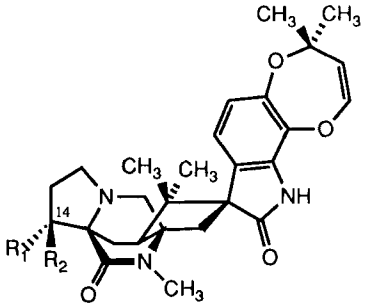
(**35-37**)

	R_1	R_2	R_3	R_4
18	F	H	$CH(CH_3)_2$	H
19	OCH_3	H	$CH(CH_3)_2$	H
20	OH	H	$CH(CH_3)_2$	H
21	NH_2	H	$CH(CH_3)_2$	H
35	CH_3			
36	CH_2CH_3			
37	$CH(CH_3)_2$			
38	H	OH	$CH(CH_3)_2$	OH
39	OH	H	$CH(CH_3)_2$	(*t*-bu)Me_2Si

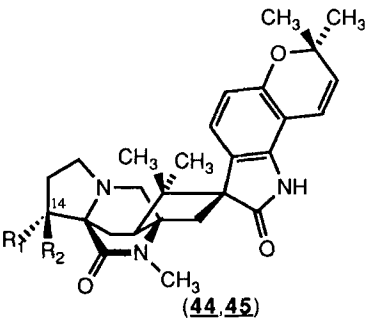
Mode of action - The mode of action of the avermectins/milbemycins has remained elusive, at least in part due to the fact that avermectins have been studied in so many different model systems with an array of experimental protocols (see reference 23 for a recent review). It is now well established that avermectins specifically open a chloride channel in invertebrate systems

distinct from the GABA-gated chloride channel (24,25) and a specific, high affinity avermectin binding site has been characterized in a non-parasitic nematode (26,27).

Other anthelmintics - Paraherquamide (**40**) is an indole alkaloid first isolated from *Penicillium paraherquei* (28). Recently, paraherquamide was identified as a potent nematocidal agent with anthelmintic activity against immature *Trichostrongylus colubriformis* in a rodent model system (29). Paraherquamide is highly efficacious in sheep as a single oral dose against *Haemonchus contortus, Ostertagia circumcincta, T. colubriformis* and *Cooperia curticei* (30). Ivermectin-resistant and thiabendazole-resistant nematodes are sensitive to paraherquamide treatment *in vivo* suggesting a different mode of action (30). A large number of naturally occurring and semi-synthetic analogs (**41**-**45**) were identified and evaluated for anthelmintic activity, with only the C-14 position analogs retaining biological activity (31-33).



(**40**-**43**)



(**44**,**45**)

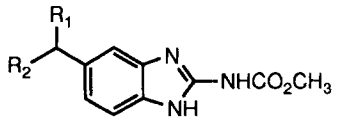
	R_1	R_2
40	OH	CH_3
41	H	H
42	=CH_2	
43	H	CH_3

	R_1	R_2
44	H	CH_3
45	OH	CH_3

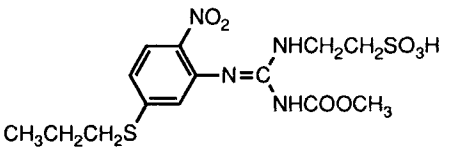
A series of methyl 5-substituted benzimidazol-2-yl carbamates was synthesized and structure-activity relationships were delineated with respect to antifilarial activity. Compounds 46 and 47 displayed excellent activity against *Brugia pahangi* in jirds (34,35), while certain fluorine containing 9H-1,2,4 triazolo-4 3-A-benzimidazoles were found to have good insecticidal activity (35).

Netobimin (**48**) is a broad-spectrum anthelmintic having both facsciolicidal and nematocidal properties (36). It is efficacious at 20 mg/kg in sheep experimentally infected with *F. hepatica* (adult and immature stages), and is also active against trichostrongyloid gastrointestinal nematodes.

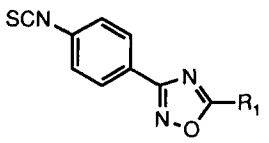
A series of 3- and 5-(isothiocyanatophenyl)-1,2,3-oxadiazoles was synthesized and tested for nematocidal activity. The two most active members of this series (**49** and **50**) were active against the gastrointestinal nematodes of sheep at 100 mg/kg. In addition, **50** was also active against hook worms in dogs at a single oral dose of 200 mg/kg (37).



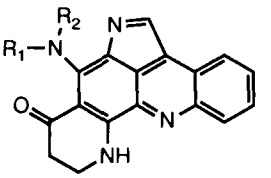
	R_1	R_2
46	$NHCH_3$	C_6H_5
47	$NHCH_2CH_3$	H



48



49 $R_1 = H$
50 R_1 = furan



	R_1	R_2
51	H	CH_3
52	CH3	CH_3

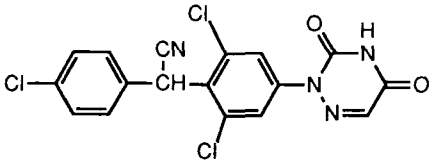
Two novel pentacyclic aromatic alkaloids, plakinidine A and B (**51**, **52**, respectively) were isolated from a *Plakortis* sponge (38). Both compounds exhibited *in vitro* activity against *Nippostongylus brasiliensis*.

PROTOZOAL DISEASE

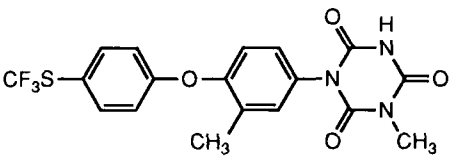
Antiprotozoal Agents - The development of new chemotherapeutic agents for the treatment and control of protozoan infections has been limited over the last decade due to a strong interest in the development of vaccines especially in the area of malaria and coccidiosis. Since viable vaccines have yet to be developed, there is a renewed interest in drug discovery.

Coccidia - Coccidiosis in poultry is the major area of interest for anticoccidial agents. Polyether ionophores continue to be the choice of most poultry producers, however resistance to the entire class is becoming more common and the therapeutic window remains narrow. Two new closely related ionophores currently being evaluated for the broiler industry, maduramicin and semduramicin, are more potent then previous ionophores, but may be ineffective against ionophore resistant coccidia. In response to ionophore resistance, a new combination agent has been introduced which contains the ionophore narasin and the older anticoccidial nicarbazin. These two agents appear to synergize which allows for a reduction in the combined use level. There is concern that coccidia resistant to this combination will be difficult to control since nicarbazin has traditionally been used to control outbreaks of ionophore resistance. One of the newer approaches for preventing the development of drug resistance involves the use of shuttle programs, where different anticoccidial agents are used sequentially.

The triazinones are the only class of non-ionophore anticoccidials introduced in the 1980's. Two compounds in this class have been introduced in overseas markets, diclazuril (**53**) and toltrazuril (**54**). Diclazuril has an optimal use level of 1 ppm, broad spectrum activity and is well tolerated (39, 40). Organisms resistant to diclazuril have been produced in studies designed to evaluate resistance frequency (41).



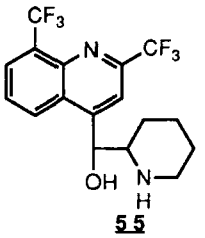
53



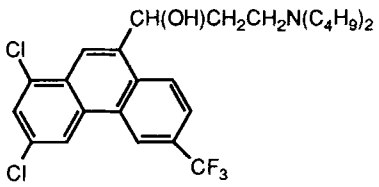
54

A new book on the coccidia has been published which includes chapters reviewing the chemotherapy of coccidiosis and progress toward vaccine development (42).

Plasmodia - Drug resistance remains a major problem with treating malarial infections. Recent studies have demonstrated that agents capable of reversing multidrug resistance, such as desipramine or verapamil, can reverse the drug resistance of malaria parasites to chloroquine. (43,44). It is believed that multidrug resistance occurs in many cases as a result of the selective efflux of drug by the parasite through a multidrug resistance transport system.



55



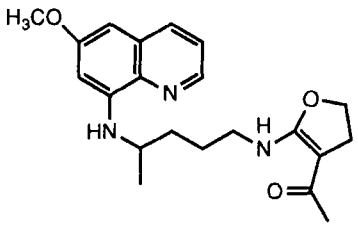
56

Mefloquine (55) is currently being used for the treatment of malaria. The compound is active against chloroquine resistant organisms and is being introduced as a single entity and in combination with other agents in an attempt to delay the development of resistance. However, resistance to mefloquine is already beginning to occur (45,46). Another compound, halofantrine (56) is currently in phase III trials. It may be ineffective against some strains of mefloquine resistant malaria (46). Clinical efficacy has been demonstrated in patients that failed chloroquine and/or quinine therapy. The drug is effective and well tolerated in the clinic (47-49) with the exception of some cases of incomplete clearance of parasites after an initial treatment (50).

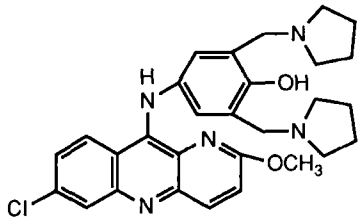
An analog of primaquine, CDRI 80/53, (57) has been shown to have curative antirelapse activity against *Plasmodium cynomolgi* in the monkey (51) and is considered to be safer than primaquine in mice and dogs (52, 53).

Pyronaradine, a 9-substituted 1-aza-acridine (58), is currently being evaluated as a new antimalarial agent. Mefloquine resistant field isolates are susceptible to this agent (54) however, cross-resistance with chloroquine may occur (55,56).

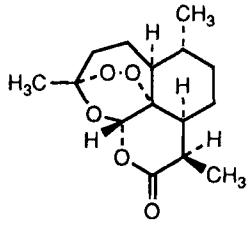
Artemisinin (59), a sesquiterpene lactone isolated from a Chinese herb, has been the focus of recent antimalarial drug development efforts. Dihydroartemisinin (60) is produced by sodium borohydride reduction of 59 and is more potent than the parent compound. Both compounds are effective *in vivo* against chloroquine resistant organisms and are efficacious against cerebral malaria. Artemisinin has also been shown to synergize with mefloquine and tetracycline while pyrimethamine and chloroquine were found to be antagonistic with artemisinin (57). Water soluble analogs of dihydroartemisinin have been produced. One of the early derivatives, sodium artesunate (61), was effective against malaria *in vivo* but was found to have



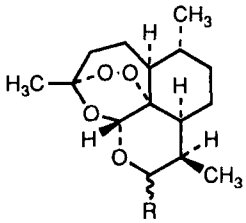
57



58



59



60 R= OH

61 R= α $OCOCH_2CH_2CO_2Na$

62 R= ß $O-CH_2-C_6H_4-COOH$

63 R= H

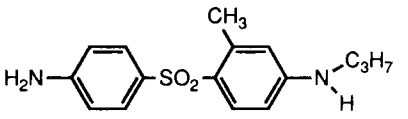
stability problems due to hydrolysis of the ester linkage; further chemistry led to water soluble artelinic acid (**62**) which was stable and superior in activity (58). Recently, dextro-deoxoartemisinin (**63**) was prepared from artemisinin in one step and found to have superior *in vivo* activity (59). All of the members of this class lack oral activity and are limited to parentral use. These compounds have gametocytocidal activity indicating that treatment with this class may be useful in preventing the transmission of malaria (60).

Some orally active iron chelators exhibit antimalarial effects *in vivo*. It has been suggested that these compounds deprive the parasite of its iron requirements without effecting the plasma iron levels of the host (61-62).

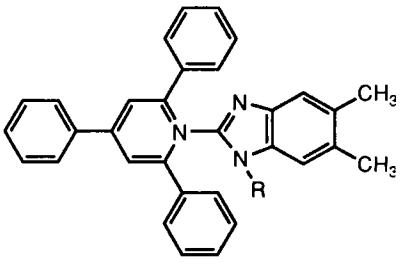
A series of polyamine analogs with the general formula $RNH(CH_2)_xNH(CH_2)_yNH(CH_2)_xNHR$ has been synthesized and tested against *Plasmodium falciparum in vivo* and *in vitro* (63). The optimum chain length for antimalarial activity was x=3, y=7. The most potent compound with x=3, y=7 and R=benzyl was fully effective in mice at 15 mg/kg. All of these compounds were tested in combination with DL-α-difluormethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (63).

A dapsone analog (**64**) has been synthesized and shown to be effective against malaria when used in combination with 7,8-dihydrofolate reductase inhibitors (64). This compound may be much safer than dapsone based on the lack of methemoglobin production at doses as high as 200 mg/kg.

Pentamidine analogs have been reported which retain their antimalarial activity and may have reduced levels of toxicity to the host (65).



64



65 R = H
66 R= CH_3

Leishmania and Trypanosomes - A new class of agents, pyridinium azolate betaines and their derivatives (**65**, **66**), show antileishmanial activity *in vivo*, and also possess *in vitro* activity against *Trypanosoma cruzi* (66).

In vivo antileishmanial activity has been demonstrated with the hydrazide monoamine oxidase inhibitor antidepressants, phenelzine and nialamide. Relatively high doses were required to achieve *in vivo* activity with these compounds (90 mg/kg) (67).

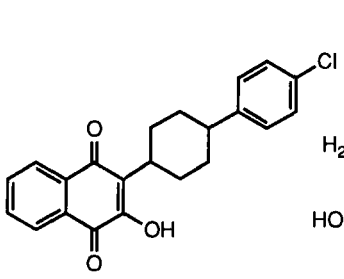
Studies evaluating the antitrypanosomal effects of DFMO continue in humans. Effective treatment of Gambian trypanosomiasis has been demonstrated with minimal side effects and the compound has been found in cerebrospinal fluid, demonstrating CNS penetration (68). A more potent analog of DFMO, monofluoromethyldehydroornithine methyl ester, was found to be accumulated by trypanosomes 6-8 times faster than DMFO, suggesting that a lower dose and/or shorter regimen may be required in humans (69).

Several mechanisms currently being explored as potential targets have recently been reviewed (70). These include purine and pyrimidine salvage and biosynthesis, polyamine biosynthesis, microtubule assembly, transcription of messenger RNA, and the biosynthesis and function of a metabolite of trypanosomes.

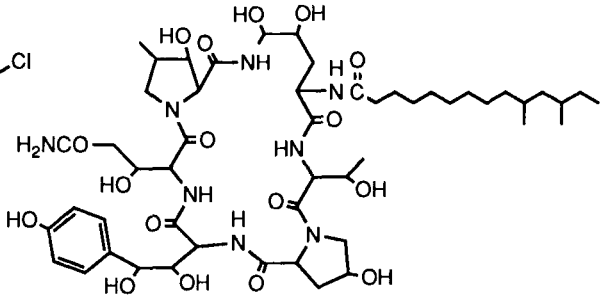
Other Protozoan Parasites - There has been a renewed focus on several organisms as a result of the AIDS epidemic. The severe immunocompromised state of these patients has resulted in the

appearance of *Pneumocystis carinii, Toxoplasma gondii* and *Cryptosporidium parvum* as life threatening infections.

Pneumocystis - *Pneumocystis carinii* pneumonia remains one of the major causes of death in AIDS patients, and prophylaxis with aerosolized pentamidine or low doses of trimethoprim-sulfamethoxazole or dapsone are currently being used to prevent the onset of the disease. It is still not clear whether this organism is a fungus or protozoan. DNA sequence homology of the ribosomal RNA gene (71, 72) and studies of the enzyme thymidylate synthase (73) suggest that it is a fungus; however, it has been found to be sensitive to many known antiprotozoal agents and insensitive to all of the currently used antifungals. Two additional antiprotozoal agents have been reported to show activity against *P. carinii* including several 8-aminoquinolines (74) and 566C80 (**67**) a hydroxynapthoquinone originally developed for treating malaria (75). L-671,329 (**68**), a ß-1,3 glucan synthesis inhibitor with a narrow spectrum of antifungal activity has been shown to have potent antipneumocystis activity (76). This is the first report of an antifungal compound with activity against *P. carinii*.



67



68

Toxoplasma - No new therapies have been described for this organism. Currently, either spiramycin or pyremethamine-sulfamide is used. The antimalarial hydroxynapthoquinone, 566C80, has also been reported to have *in vivo* activity against toxoplasmosis in mice at 50 mg/kg making it potentially useful for treating both toxoplasmosis and pneumocystis in AIDS patients (77).

Cryptosporidia - There is an urgent need for new agents to treat cryptosporidial infections. *Crytosporidium parvum* is capable of continuously reinfecting the gut of the immunocompromised patient resulting in constant diarrhea and associated weight loss. There is currently no useful therapy for treating this condition. Diclazuril (**53**) is being evaluated in AIDS patients to determine it's potential use for treating cryptosporidiosis, with early indications that it is ineffective (78).

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Chapter 18. Function of Human Retrovirus Regulatory Proteins.

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Introduction - The rapid onset of the Acquired Immune Deficiency Syndrome (AIDS) pandemic best illustrates that the human retroviruses represent a formidable group of pathogens. The human T cell leukemia viruses, HTLV- I and HTLV- II, are associated with adult T cell leukemia/lymphoma and a more benign form of hairy cell leukemia, respectively. The human immunodeficiency virus (HIV), a member of the lentivirus family, is the etiological agent of AIDS. Both families of viruses infect human T4 lymphocytes, with HTLV-1 infection leading to transformation while infection with HIV results in cell death. The complex genomic organization characteristic of these viruses sets them apart from the prototype murine and avian retroviruses. All retroviruses contain three genes encoding the necessary replication functions; the *gag* gene encodes the capsid protein surrounding the RNA genome; the *pol* gene encodes the proteins necessary for reverse transcription and integration; and the *env* gene encodes the glycoprotein necessary for binding and fusion with the target cells. In addition, the viral genome is flanked by sequences called long terminal repeats (LTRs). These sequences harbor the regulatory elements similar to promoter and enhancer elements that control expression of cellular genes.

The human retroviruses encode both essential and non-essential non-structural proteins with diverse regulatory functions. In addition to their function in regulation of virus gene expression these viral transactivator proteins are thought to usurp host cell functions, ultimately leading to inappropriate expression of genes involved in cell proliferation. This review summarizes our current understanding of the mechanism of action of the predominant regulatory proteins encoded by the human retroviruses.

Human T Cell Leukemia Virus

Human T cell leukemia virus (HTLV-1) represents the first pathogenic human retrovirus identified (1). HTLV-1 infection is endemic to southern Japan, the Caribbean basin, and the southwest United States and is etiologically associated with adult T cell leukemia/lymphoma.

Infection with HTLV-1 has also been associated with tropical spastic paraparesis, a chronic demyelinating neurological disease (2,3). Claims linking HTLV-1 infection with multiple sclerosis have also been made (4). The closely related HTLV-2 virus was originally isolated from a patient with a benign form of hairy cell leukemia (5). More recent studies, using PCR analysis, indicate that a significant proportion of intravenous drug users are also be infected with HTLV-2 (6).

The genomic organization of the HTLV viruses is complex. Notably, the 3' end of the viral genome, originally designated as the X region (7), encodes at least two regulatory proteins, referred to as Tax and Rex (Fig.1) (8-11). Both are positive regulators of gene expression, with Tax functioning at the transcriptional level (12-16) and Rex functioning post - transcriptionally (16-18).

Transcriptional Regulation - The observation that Tax could transcriptionally activate gene expression provided the first clue that the human retroviruses were dissimilar to their avian and murine counterparts. Consistent with its involvement in transcriptional activation, Tax is localized in the nucleus, although a significant amount is present in the cytoplasm (9,12,19). Transactivation of LTR directed gene expression by Tax requires the presence of at least one 21bp element repeated three times within the LTR (20-25). Most studies suggest that the mechanism for transcriptional activation does not involve a direct interaction of Tax with DNA. Rather, transactivation is thought to

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occur through modification or induction of pre-existing host proteins (Fig.1). Consistent with this prediction, DNAse footprinting over the 21bp element indicates the sequence TGACGCA, identified as a consensus sequence important for induction of gene expression by cyclic AMP (cAMP), is protected (26-28). Thus, it is possible that a common nuclear factor mediates activation by cAMP and Tax. Moreover, HTLV-1 LTR directed gene expression is inducible following addition of cAMP (29,30), and purified transcription factors which bind to the cAMP responsive element also interact with HTLV-1 21bp sequence (27,28). The involvement of cellular factors in the control of gene expression by Tax may, in part, explain how the activity of cellular genes which lack the 21bp motif are Tax responsive (see below).

Post Transcriptional Regulation - It has been known for some time that the 3' region of the HTLV-1 genome encodes proteins in addition to TAX. Only recently has the function of the p27 product, now referred to as Rex, been elucidated. The Rex protein, like Tax, is nuclear but displays marked accumulation in the nucleolus (31,32). Rex post-transcriptionally regulates the expression of unspliced and single spliced mRNAs encoding the *gag/pol* and *env* genes by allowing transport of these mRNAs to the cytoplasm, permitting translation into protein and subsequent virus assembly (16-18). The Rex responsive sequence is formed by base pair interactions within RNA sequences present in the 3' LTR forming an elaborate secondary structure (33). If the mechanism of Rex action is analogous to that of the HIV Rev protein (see below) then it probably exerts its effect through direct interaction with an RNA target sequence.

Tax and Transformation - Both HTLV-1 and HTLV-2 efficiently transform human T cells *in vitro* (29,34-36). Other oncogenic retroviruses induce malignancy by insertion of their LTR near cellular oncogenes or they express a transduced oncogene, which by definition is a homologue of a cellular gene. HTLV-1 neither encodes an oncogene nor does it display site-specific integration. However, recent studies provide compelling evidence that expression of Tax is sufficient to confer immortalization. In one study, human T cells were immortalized after infection with a herpes virus vector expressing the *tax* gene (37). In a second study, transgenic mice expressing Tax developed unusual malignancies (38). The ability of Tax to activate viral gene expression suggested that transformation might be achieved if Tax can alter expression of cellular genes that control cell proliferation. This phenomenon has been established with studies demonstrating that expression of genes encoding interleukin-2 (IL-2), interleukin-2 receptor (IL-2R) (39-43) and granulocyte macrophage colony stimulating factor (GM-CSF) (44,45) is elevated in the presence of Tax. Consistent with these *in vitro* observations, HTLV-1 transformed cell lines and ATL tumor cells have been observed to constitutively express both IL-2R and GM-CSF (35,46-48).

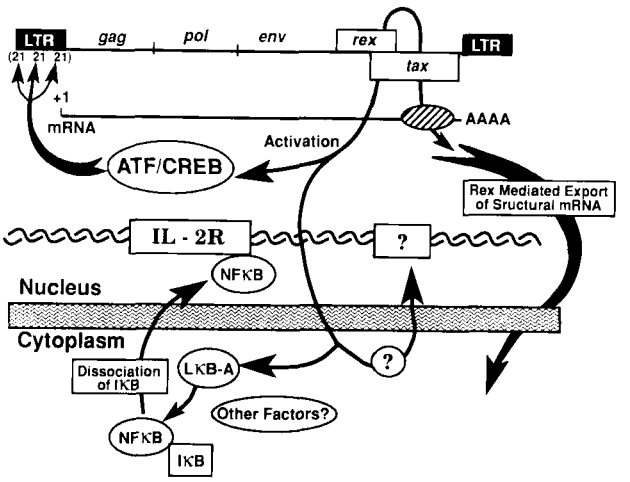


Fig. 1. Expression and function of human T cell leukemia virus regulatory proteins. The diagram illustrates the potential interaction of HTLV-1 regulatory proteins, Tax and Rex, with host cell proteins, ultimately leading to regulation of viral and cellular gene expression.

The mechanism of Tax-mediated activation of cellular gene expression appears to be similar to that employed for activation of the LTR, with the exception being that different cellular factors are probably involved. Activation of the IL-2R gene requires the DNA sequence, GGGGAATCTCC, which differs from the Tax responsive sequence present in the LTR. However, the IL-2R Tax responsive element does bear striking similarity to the NF-κB binding motif present in the immunoglobulin kappa light chain enhancer and has been shown to bind NF-κB (49-51). Results from other studies indicate that activation of NFkB is achieved by dissociation of a repressor molecule, IkB, which then permits translocation of NFkB to the nucleus (52,53). In cell lines which constitutively express Tax, activation of NFkB proceeds via activation of a pre-existing factor, designated LkB-A, present in cells of lymphoid lineage (Fig.1) (54). Thus, the events leading to activation of NFkB by Tax are post-translational. In this respect, Tax joins the growing list of transcription factors that regulate gene expression through protein-protein associations (55-58). The mitogens PHA and PMA and the cytokine tumor necrosis factor also activate NFkB through a post-translational mechanism (52,53,59-61). Therefore, it is possible that mitogenic stimulation, through activation of protein kinase C, leads to activation of NFkB by the same pathway utilized by Tax. The hypothesis that expression of Tax leads to activation of cellular gene expression through a cascade of events involving protein-protein associations, rather than direct interaction of Tax with DNA would explain the dissimilarity among the many Tax responsive DNA sequence elements. If Tax interacts with multiple proteins, the ability to do so may be conferred by different functional domains.

The ability of Tax to function through different protein-protein associations predicts that the outcome of Tax expression will be dependent upon the constituents present in any one cell lineage. In this respect, tissue specific expression of host-encoded transcription factors could be the major determinant governing the ability of Tax to regulate expression of cellular genes. This may explain the diverse clinical manifestations observed following infection with HTLV-1 or expression of Tax in different tissues.

Human Immunodeficiency Viruses (HIV)

The pathological outcome of infection with HIV is depletion of the T4 lymphocyte population. Consequently, AIDS is characterized by severe infection with opportunistic organisms resulting from the immunodeficiency. Whether the high frequency of malignancies, such as Kaposi's sarcoma or B cell lymphoma, results from immune suppression or the action of a specific viral gene product is currently the topic of intense investigation.

Significant progress toward understanding the complexities of HIV gene expression has been made. With respect to genomic organization and regulation of gene expression, HIV is probably the most complex retrovirus studied to date (Fig.2). For simplicity, regulation of gene expression can be divided into three levels. First, cellular transcription factors such as NFkB and SP1 and regulatory proteins encoded by several members of the herpes virus family influence HIV gene expression (62-65). Second, HIV encodes two positive regulators of gene expression, referred to as Tat (66,67) and Rev (68,69) both of which are essential for replication and thus represent attractive targets for prophylactic and therapeutic intervention. Finally, there exists a class of proteins that serve accessory functions affecting replication and infection, but which are not essential.

Tat - Tat is the best studied of the viral transactivator proteins. Originally described as a transcriptional activator protein, it is now thought that Tat functions at several levels to control gene expression. The 86 amino acid Tat protein, like the Tax protein of HTLV, is produced from a doubly spliced mRNA formed by joining an exon preceding the *env* gene with a second exon within *env*. Mutational analysis suggests the presence of at least three functional domains within the Tat protein. An acidic group of amino acids present at the extreme amino terminus consisting of two glutamic residues and one aspartic acid has been suggested to have a periodicity of acidic, polar, and hydrophobic residues consistent with that of an amphipathic alpha helix (70). Although this conformation has been linked to several transcriptional activator proteins, its role in Tat function has not been clearly defined.

The basic domain is required for both nuclear and nucleolar localization (71,72) and for the ability to interact with RNA (see below). Residues 48-52, essential for Tat's nuclear localization, also function on heterologous proteins to target them to the nucleus (72). The role of nucleolar localization, which has become a hallmark feature of the HIV and HTLV RNA binding proteins (i.e. Rev and Rex), is not yet understood.

The most striking feature of Tat is an unusual clustering of seven cysteine residues. A similar grouping of cysteines is also present in the Tat proteins encoded by HIV-2 and the simian immune deficiency viruses (SIV). The cysteine residues are required for metal ion linked dimerization, but are unlikely to form a typical zinc finger motif (73). Mutation of most of the individual cysteine residues destroys Tat function (72,74) suggesting that the ability of the cysteines to coordinate with metal serves an important function. The ability of Tat peptides lacking these cysteine residues to bind specifically with viral RNA suggests that the physiological function of metal-linked dimerization is something other than RNA binding.

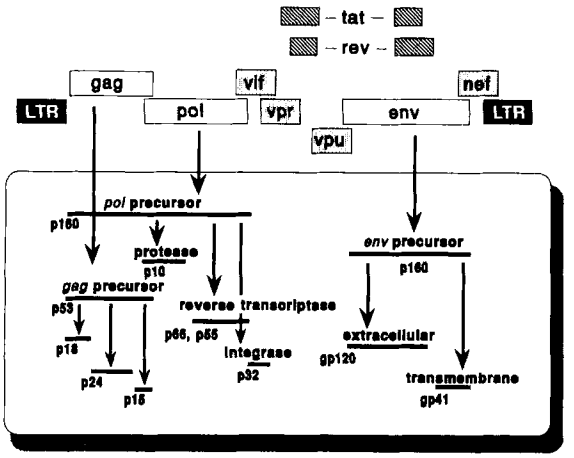
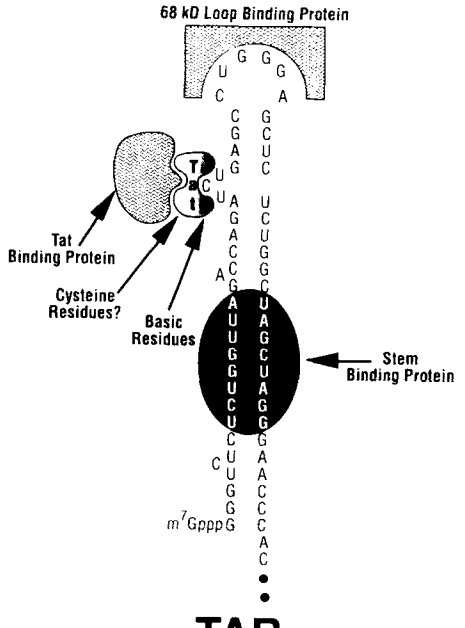


Fig. 2. Genomic Organization of HIV. The genetic composition of all retroviruses consists of three principal structural genes (*gag, pol, env*) that encode the proteins necessary for virus assembly. These structural polyproteins are cleaved during maturation of virions as shown. Unlike their avian and murine retrovirus counterparts, the human retroviruses, encode numerous non-structural proteins with diverse regulatory functions. For simplicity, the regulatory proteins of HIV can be grouped into two classes; proteins essential for virus replication, exemplified by Tat and Rev, and proteins which enhance replication and or infectivity, such as vpu, vif, vpr and nef.

Interaction of Tat with TAR RNA: mechanistic Insights - Other than being able to elicit effects at the transcriptional and post-transcriptional level, the mechanism of Tat function is poorly understood. The Tat responsive sequence, known as TAR (Fig.3), is present within the first 60 nucleotides 3' of the transcription initiation site (75-80) in the S1LTR. The location of TAR (3' to the promoter) as well as several other properties distinguish it from other well characterized transcriptional control elements. TAR function is position and orientation dependent, with maximal activity obtained when TAR is present at the extreme 5' terminus of the mRNA transcript (75,80-83). TAR clearly differs from the classical enhancer elements where function is both position and orientation independent. Function of TAR is also independent of other upstream HIV regulatory sequences although the presence of a TATA box and enhancer, that can be derived from any source, are required for transactivation. The likelihood that TAR functions as an RNA target was first suggested by the observation that a stable stem loop structure is formed by base pair interactions between nucleotides +1 to +60 (83). In other studies, mutations in the stem which alter primary nucleotide sequence without affecting secondary structure were found to fully support transactivation (76,79,81). In contrast, alteration of nucleotides in either the bulge or loop structures completely abolishes the Tat response (76,84). These findings support the hypothesis that TAR functions as an RNA target. More recent studies suggest that Tat probably recognizes the bulge region (84,85). Binding of Tat to TAR RNA is not however, in itself, sufficient for function. For example, TAR RNAs which contain mutations in the loop, although not Tat responsive, still bind to Tat (86). Since the loop structure is required for binding of a 68kDa cellular protein (87), the cumulative data imply that transactivation probably depends upon the presence of both Tat and additional cellular factors (Fig.3).

An understanding of how the interaction of Tat and/or cellular factors with TAR RNA regulates HIV gene expression has yet to emerge. Adding to the difficulty in interpreting the vast amount of data accumulated on this subject, are the diverse systems that have been used to examine Tat function. Transactivation has been studied transiently, in stable cell lines expressing Tat, *in vitro* and in cells of human and non-human origin. Evident in each of these systems is the increase in the steady-state level of LTR directed mRNA following expression of Tat. However, the discordance between Tat induction of RNA and protein suggest that processes other than transcription are also involved. To reconcile the latter observation, several hypotheses have been considered. First, one can assume that the function of Tat is at least bimodal, as has been suggested (77,88). Alternatively, if Tat function is dependent upon different host cell factors, as the data imply, then one would expect different results using different experimental systems. The most direct evidence suggesting a dependence upon host cell factors are results obtained using mouse-human hybrid cell lines. In these studies, a significant level of transactivation was only achieved in hybrid cells containing human chromosome 12, implying that a gene on this chromosome encodes a human specific factor that aids in the Tat response (89). Results from gel electrophoretic shift assays point to the existence of numerous host cell factors that interact with TAR at the level of both DNA and RNA (64,87,90-93). One model for transactivation incorporating a Tat-TAR interaction is the ability of Tat to function as an anti-terminator to block premature termination of RNA transcription within TAR. Because in these studies no increase in the overall rate of transcription initiation was observed, the data suggest that Tat may effect elongation. Since removal of TAR has little effect on the basal activity of the LTR, indicating that it is not a negative element, the genetics of this system do not completely support the anti-termination model, but do not rule out a role of Tat in elongation. Results obtained in other studies, showing that the abundance of short transcripts present in the absence of Tat does not change in its presence, are consistent with *in vitro* transcription experiments suggesting that these transcripts may arise from co-transcriptional processing of RNA within TAR (94).



In several studies Tat has been shown to exert a clear post-transcriptional effect; the best example being studies in Xenopus oocytes. When heterologous RNA transcripts containing the TAR region are injected into the nucleus of Xenopus oocytes, transactivation is obtained in the presence of Tat (95). Moreover, this occurs in the presence of transcriptional inhibitors. Since Tat has no effect when co-injected with TAR RNA in the cytoplasm, these studies imply that in Xenopus oocytes the function of Tat is independent of transcription and may affect the translational competence of nuclear TAR RNA. However, when purified, functional Tat protein is added to mammalian cells in the presence of transcriptional inhibitors, no transactivation is observed (96). Thus, in this system, the data do not support a mechanism were Tat interacts with TAR RNA to affect its transport, stability, or translation. These discrepancies may indicate that Tat functions at several levels of control, and imply that factors involved in transactivation in Xenopus may be unique to this system.

Fig.3. RNA sequence elements recognized by Tat and cellular factors. The putitive protein binding domains on the Tat responsive TAR element are shown.

Although Tat's potential function in elongation and post-transcriptional effects cannot be dismissed, there is another, more intriguing suggestion that the Tat-TAR interaction serves as an

RNA enhancer (97,98). Such a mechanism, which has yet to be described in other systems, would be analogous to that of a DNA enhancer except that interaction of Tat with TAR RNA, rather than DNA, would facilitate an increase in transcription initiation. If the interaction of Tat with TAR RNA serves this function, it may facilitate formation of a transcription complex at the promoter, the enhancer, or both. Similar mechanisms of cooperativity are thought to exist between promoter and enhancer factors to enhance transcriptional activity. If the interaction of Tat with TAR has the same effect, the positional requirement for TAR at the 5' terminus of the transcript suggests that the Tat-TAR interaction must occur in proximity to the promoter. Therefore, although producing the same effect as a DNA enhancer, the RNA mediated enhancement of transcription would have less flexibility. If such a model is correct, then it is likely that interaction of Tat and /or TAR with cellular factors is required for this process. Consistent with this prediction is the observation that Tat associates with at least one nuclear protein, designated TBP-1 (99). Although the role of the TBP-1 interaction has yet to be determined, it is tempting to speculate that the interaction of Tat with TBP-1 and/ or other cellular factors forms part of the transcriptional initiation or elongation complex involving multiple interactions between protein, DNA and RNA.

Modulation of Cellular Gene Expression by Tat - Most recent studies support a direct role for Tat in regulation of cellular processes. Studies with transgenic mice expressing Tat provide clear evidence for such additional functions (100). Some of these animals develop a syndrome similar to Kaposi's sarcoma, a malignancy prevalent among HIV infected individuals (100). Tat has also been found to enhance the growth of Kaposi's sarcoma derived tissue in culture (101). This latter observation, together with the absence of Tat in affected cells, supports the hypothesis that Tat may behave as a growth factor or cytokine. Although this has yet to be proven, *in vitro* studies show that Tat is readily taken up by cells when placed in the extracellular environment (96,102). Addition of Tat to culture medium has also been found to suppress antigen induced proliferation of T lymphocytes (14). As this observation closely parallels that seen in infected individuals, it has been suggested that circulating Tat, if it does exist, may contribute to the immunosuppression associated with AIDS.

Rev, an Antirepressor Protein - Rev represents the second essential HIV regulatory protein (68,103). A clue to Rev's existence first came from a phenotypic observation that proviral mutants containing frame shift deletions in the region now known to encode Rev did not yield detectable levels of *env* or *gag* gene products (68,103). It is now thought that Rev mediates the export of nuclear entrapped, viral structural mRNA to the cytoplasm. If one divides HIV gene expression into early and late phases, analogous to that observed with DNA tumor viruses, the following assumptions can be made. In the absence of Rev, viruses would be unable to progress to the structural phase of gene expression (late) and would remain trapped in the regulatory phase of replication (early). Indeed, evidence exists that early in infection one sees expression of the regulatory proteins Tat , Rev and Nef, which is then followed by the appearance of incompletely spliced transcripts encoding the structural gene products (104).

The Rev protein, like Tat, is an essential gene product that is conserved among various members of the lentivirus family. The genes encoding Rev and Tat overlap, with each protein being produced from a different reading frame. Rev is a 19 kDa nuclear phosphoprotein (105-107) found almost exclusively in the nucleolus (78,108,109). Mutations that eliminate the ability to localize in the nucleolus also abolish function (110). Although the role of nucleolar localization is obscure, studies from other systems, which show that nucleolar proteins shuttle to the cytoplasm, would be consistent with Rev's involvement in mediating transport of HIV structural mRNAs.

There is general agreement that Rev, through an interaction with an RNA structure (RRE; Fig.4) (see below) serves to activate gene expression. It probably does so by indirectly overcoming negative effects exerted by other regions of the HIV genome. Early studies demonstrated the existence of *cis*-acting negative elements (termed CRS sequences) dispersed throughout the HIV genome which suppress gene expression in Rev's absence (111-113). Other studies suggest that inefficient splicing of HIV transcripts, which results in nuclear accumulation of unspliced precursors, is responsible for the lower level of expression of the structural genes (111,114-116). This phenomenon can be demonstrated in an artificial Rev dependent system involving hybrid globin RRE transcripts, lacking either a functional splice donor or acceptor sequence (117). However, Rev can act on mRNA that lacks functional splice sites. To reconcile these potential differences, it can be hypothesized that factors which bind to inefficient or mutated splice sites (exemplified by the hybrid globin-RRE RNA) and possibly cryptic splice sites within the CRS elements, elicit nuclear entrapment of the HIV structural mRNA. The mechanism for nuclear entrapment of the HIV structural mRNA is clearly an area in need of further study.

Whatever the mechanism of entrapment may be, there is agreement that the Rev response element, termed RRE, through an interaction with Rev, functions independently of these "negative" elements to mediate transport of the nuclear entraped mRNA to the cytoplasm. The RRE element, originally refered to as CAR for cis-acting anti-repression sequence (111-113,115-116,118) forms a complex RNA secondary structure (Fig.4) (115). Mutational analysis supports the existence of secondary structures within this region (119,120). Most recent studies have established that Rev interacts with RNA containing the RRE sequence (120-123). So far, the data suggest that RNA secondary structure, as opposed to primary nucleotide sequence is the major determinant for this interaction (119). In support of this hypothesis, compensatory mutations that maintain secondary structure, but alter primary nucleotide sequence in the stem loop structures found to be required for Rev interaction, still bind to Rev and are functional *in vivo*. Similar findings have been obtained with HIV-2 Rev protein which interacts with RNA secondary structures generated within HIV-2 RRE RNA (124). Further investigations should answer whether some degree of primary nucleotide sequence, in addition to secondary structure, is required for the Rev-RRE interaction.

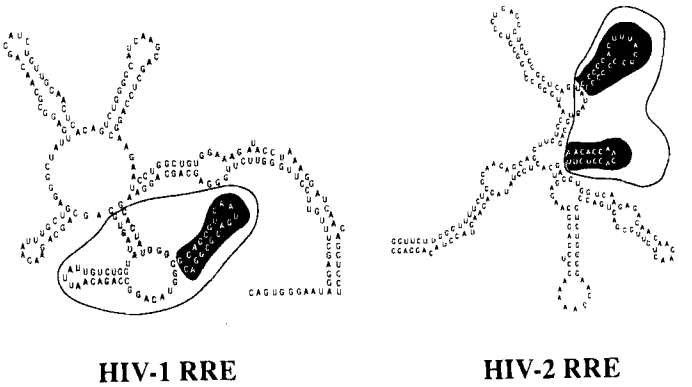


Fig. 4. Schematic illustration of the Rev responsive element (RRE) RNA structures. The computer predicted and experimentally confirmed structures for the RRE elements of HIV-1 and HIV-2 are shown. The putative Rev binding site within each RRE is illustrated by shading.

Transdominant Rev Mutants - The ability of Rev to interact with RRE RNA is not sufficient in itself to restore gene expression. There exist Rev mutants which bind to RRE-RNA, yet do not restore gene expression. Co-transfection studies have shown that mutation of amino acids 78 and 79 of Rev produce a protein which functions as a transdominant suppressor of Rev function (69). Studies with additional mutants in this region (125), as well as mutations within the same region of the HTLV-1 Rex protein (31), suggest that this region in both proteins functions as an activation domain. RNA binding studies with these HIV transdominant suppressor mutations demonstrate that they form a stable interaction with RRE-RNA comparable to that obtained with authentic Rev protein (125). Thus, the transdominant suppression is not attributable to a failure to interact with RRE-RNA. The inability of the transdominant Rev proteins to restore gene expression, yet interact with RRE-RNA, indicates that binding alone is insufficient for function. This suggests that binding of additional cellular factors to either Rev or the Rev-RRE complex is necessary for function. Thus, the accumulated data suggest that at least two steps are required for Rev function; association of Rev with RRE-RNA, followed by interaction of cellular factors with the Rev-RRE complex to mediate the productive export of structural mRNA from the nucleus to the cytoplasm. Identification of the putative host cell factors involved in the latter part of this pathway awaits further study.

Conclusion - With the incidence of both HTLV-1 and HIV infection on the rise worldwide, new approaches will be required to combat the spread of disease. Current approaches toward the development of anti-viral therapies against HIV include the use of nucleoside analogs, such as ddA (AZT), ddC and ddI, which are directed against the HIV reverse transcriptase, the use of soluble CD4 to block HIV infection, and the development of whole virus and virus sub-unit vaccines designed to prevent viral infection. These approaches have resulted in mixed success in both

laboratory and clinical trials due to problems of toxicity associated with the reagents and the relative ineffectiveness of current vaccines to provide a lasting protective immunity. It is unclear if these and other problems can be overcome. Therefore, it is important to examine additional avenues of research toward the development of safe and effective therapies against HIV. The essential viral functions served by both Tat and Rev make them attractive targets for prophylactic and therapeutic intervention. Interaction of these proteins to RNA targets is just one part of the complex viral gene expression pathways which also involve interaction with host cell factors. As *in vitro* systems are developed that allow host components to be identified and analyzed, one can begin to sort out the many interactions controling HIV and HTLV-1 gene expression. Analysis of the function of the human retroviral gene products has already provided novel insight toward the understanding of unique regulatory pathways. These insights will hopefully translate into new approaches effective against viral replication.

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SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

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Chapter 19. Chemical Basis for Immune Mediated Idiosyncratic Drug Hypersensitivity

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Introduction - Idiosyncratic drug reactions are by definition unexpected and unpredictable. While they affect a relatively small number of patients, adverse drug reactions can be fatal, and are therefore of considerable concern. Estimates for the proportion of adverse drug reactions that are immune-mediated run as high as 50%. A considerable number of these reports are based on clinical observations of symptoms rather than a definitive demonstration of drug or metabolite specific antibodies or immune lymphocytes in the patient. This chapter emphasizes only those classes of compounds for which there is significant evidence of an immune-mediated etiology. Drugs for which early reports of immune-mediated reactions were not confirmed by more recent, intensive investigations have been excluded. Definitive structure/toxicity relationships are often hard to discern, but these specific examples do illustrate some underlying principles of idiosyncratic adverse drug reactions. For further information on specific topics, the reader is refered to several recent reviews (1-11).

IMMUNOLOGY OF HYPERSENSITIVITY

Antigen Processing/Presentation - In order to stimulate an immune response, an immunogen must first be taken up by an antigen presenting cell (APC). These cells are predominantly macrophages, B lymphocytes, and Langerhans cells. Once the antigen has been endocytosed, it is "processed" in a way which is not fully understood and becomes complexed with a Class II major histocompatibility complex (MHC) glycoprotein on the APC surface. The helper T lymphocyte recognizes the foreign antigen-MHC complex, becomes activated, and sets off a cascade of events resulting in humoral (antibody mediated) and/or cell mediated immunity to that immunogen. It has recently been discovered that only 0.03 - 0.1 % of the MHC molecules on the APC need to be complexed with foreign antigen to stimulate T cell activation in a sensitized cell population (12, 13). Consequently, only minute amounts of immunogen may be required to elicit drug hypersensitivity in an individual. Furthermore, cell surface Class II MHC glycoproteins are coded for by over fifty alleles making this a highly polymorphic system. Since antigen presentation is an important controlling feature of the immune response, and since this phase of the response is determined by Class II MHC glycoproteins, the polymorphic nature of this system may be a major contributor to the idiosyncrasy of drug hypersensitivity reactions. Genetic regulation of susceptibility to hypersensitivity reactions has been demonstrated for hydralazine (14) and nickel sulfate (15). In addition, other genes lying outside the MHC also bear on the susceptibility of individuals to hypersensitivity reactions. For example, both the control of IgE production and the degranulation of basophils have been shown to be genetically determined by genes lying outside the MHC complex (16-18). The contribution that genetic polymorphism makes to the idiosyncrasy of adverse drug reactions is unquantified, but it has the potential to play a significant role.

Hypersensitivity Responses - Immune mediated hypersensitivity responses were originally categorized by Coombs and Gell into four types (19). Type I reactions are the result of IgE antibodies bound to blood-borne basophils and their tissue fixed counterparts, the mast cells. The IgE antibodies are bound by specialized receptors on the cells for the Fc portion of the

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immunoglobulin (Fc receptors) leaving the combining site free for interaction with its specific antigen. Cross-linking of the cell-bound IgE by a multivalent antigen results in an explosive release of vasoactive amines, phospholipase(s) and other inflammatory mediators from granules within these cells. If this response is systemic, anaphylaxis can ensue, and if untreated, can result in death. Hives, hay fever and some forms of asthma are common examples of this class of hypersensitivity. Type II reactions are also mediated by antibodies, but in this case IgG and IgM antibodies are responsible for the hypersensitivity reaction. Unlike the Type I response involving IgE antibodies, the IgG and IgM immunoglobulins bind to the target cell by their antibody combining site. Following recognition and binding to the individual's own cells, destruction can proceed by either complement activation, phagocytosis or by a process involving killer or "K" cells called antibody dependent cell mediated cytotoxicity (ADCC). Blood transfusion and Rh incompatibility reactions are examples of this type of hypersensitivity. Type III reactions occur when antibodies of any class bind their antigen in the blood stream and form complexes which are deposited in tissue. The resulting immune complexes fix and activate the complement cascade generating chemotactic factors such as C5a stimulating immigration of polymorphonuclear leukocytes into the area. Release of lysosomal enzymes and reactive oxygen intermediates from infiltrating cells results in tissue destruction and vasculitis. Well known examples of this type of response is the Arthus reaction, serum sickness and glomerulonephritis. Type IV reactions do not involve immunoglobulins, but instead are produced by the cell mediated arm of the immune response. They are also referred to as delayed type hypersensitivity (DTH) reactions since the peak response is observed 24 to 48 hours after antigen challenge. Type IV responses occur when specific T lymphocytes recognize antigen presented on an APC and respond by releasing a host of soluble mediators. Some of these T cell mediators help recruit and activate mononuclear cells which differentiate into macrophages capable of secreting their own inflammatory mediators. Contact dermatitis from poison ivy and positive response to tuberculosis skin testing are good examples of this type of hypersensitivity reaction.

Drug Hypersensitivity Reactions - Two other parameters of immune stimulation must be considered regarding drug hypersensitivity responses: carrier effect and multivalency. A 1000 dalton limit has been proposed as the smallest entity that can stimulate an immune response by itself (7, 10, 20). The basis for this size limitation is related to the fact that production of IgG and IgE antibodies by B cells requires activation of *both* T and B cells. Since small molecules and their metabolites are not recognized by the T cell receptor (TCR), they must first covalently couple to a larger, more complex "carrier" molecule which the T cell can recognize. In the context of the whole immunogen, the small molecular weight drug or metabolite is referred to as the "hapten". The second criterion which must be met to initiate and propagate a hypersensitivity response is multivalency. The hapten can couple to either a soluble protein or directly to a cell, but in either case, the antigen formed by these reactions must be multivalent; several hapten molecules must bind to a single protein or cell surface. Cross-linking of immunoglobulin and TCR's is imperative for initiation of immune responses, and cross-linking of cell-bound IgE is required for degranulation of mast cells and basophils. Most drugs and their metabolites are too small to accomplish cross-linking on their own, and must therefore conjugate to a carrier protein or cell in high enough density to stimulate an immune reaction.

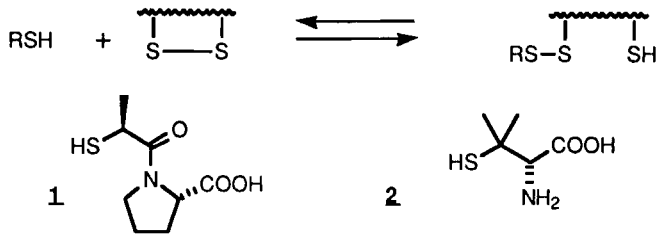
Haptenic Metabolites - Although drug metabolism is extensively studied prior to clinical trials, identification of reactive products capable of provoking an immune response is far from obvious, especially in new classes of compounds. Physiologically relevant metabolites have been generated by incubating drugs with mouse or human liver homogenates (21-23). Circulating leukocytes are also capable of converting drugs into reactive species (10, 24, 25). While generation of metabolites with liver homogenates and activated leukocytes are probably the best approaches at present, there are two caveats which should be noted. First, mouse and human liver microsomes are not necessarily equivalent and caution should be exercised when using the murine material (21). Second, it has been estimated that approximately 30% of the enzymes used for detoxification in humans are genetically polymorphic (9). In an outbred human population, therefore, one would expect individual differences in drug metabolites or proportions of metabolites. This fact in itself is most likely a contributing factor to the idiosyncratic nature of drug hypersensitivity.

Methodology - For an adverse drug reaction to qualify as immune-mediated, hapten-reactive antibodies or cells must be demonstrated in sensitized test subjects using one or more of the assays discussed below. Each assay requires an appropriate antigen, but since the natural hapten-carrier protein(s) can be difficult to identify and isolate, artificial reagents which mimic these conjugates are often prepared from readily available reagents such as Sepharose beads. The choice of hapten functional group used for coupling to the solid support is usually dictated by an understanding of likely metabolic pathways. Once a reagent is obtained, it can be used in a variety of tests to

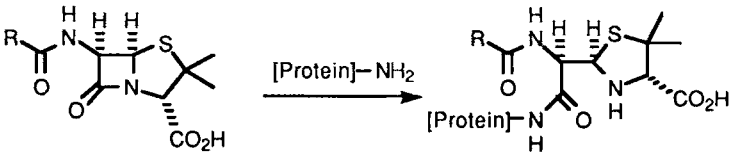
determine if the mediators of hypersensitivity reactions are present in the serum or cell population of test subjects. The radioallergosorbent assay (RAST) and enzyme linked immunosorbent assay (ELISA) are most commonly used to test for antibodies which stimulate Type I, II, and III reactions (2, 7). The hapten containing reagent is first incubated with serum from a hypersensitive individual, followed by a second detection antibody. In the case of the RAST assay, the detection reagent is radiolabeled anti-antibody. For ELISA, the second antibody is enzyme labeled, generally with either alkaline phosphatase or horseradish peroxidase. The immunoglobulin isotype of the anti-drug antibody is determined by the specificity of the second detection antibody. A negative result with this test may be misleading. If the offending *in vivo* antigen is not sufficiently mimicked by the artificial conjugate, no reaction will be observed, even in the presence of significant amounts of serum anti-drug or drug metabolite antibody. In Type II reactions, where the circulating antibodies are directed against cells, the target cell type can be treated with the reactive metabolite, and the whole cells used as the solid support for either RAST or ELISA. Since Type IV reactions are initiated by cells rather than antibodies, other methods must be employed. If an animal model is available, skin testing with the putative hapten or antigen can be performed. Inbred experimental animals are particularly useful because antigen specific cells can be passively transferred from one individual to another before administration of test agent. The most common *in vitro* test for Type IV hypersensitivity is the lymphocyte transformation assay in which cells from sensitized individuals are incubated with the test antigen and DNA synthetic response is measured using tritiated thymidine incorporation (2, 7, 26). As is the case with antibody reactions, a positive response in the lymphocyte transformation is dependent on the appropriate choice of antigen.

HAPTENIZATION OF PROTEINS AND CELLS

Aliphatic Thiols - The ubiquitous disulfide bond is a key determinant of protein tertiary structure, and covalent binding by exchange reactions with compounds possessing free sulfhydryl are to be expected. Hypersensitivity reactions to drugs which covalently bind *via* sulfhydryl groups have been well documented (3). For example, the ACE inhibitor captopril (**1**) rapidly forms disulfide bonds with plasma proteins (27-29). In addition, IgG antibodies (indicative of a Type II reaction) and lymphocytes (Type IV) specific for **1** have been identified in humans (30, 31) and rats (32). In patients with adverse reactions to **1**, both *in vitro* lymphocyte transformation as well as skin reactions were demonstrated, providing additional evidence for a Type IV hypersensitivity reaction to captropril. D-Penicillamine (**2**) is another example where drug hypersensitivity may be mediated by a thiol exchange reaction. However, specific immunological responses to this drug have been convincingly demonstrated only in rats and mice (33-35).

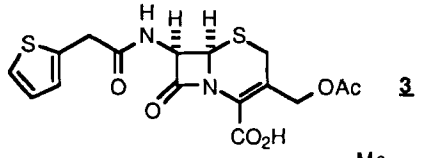


Beta Lactams - There is conclusive evidence for the occurrence of immunologically mediated hypersensitivity reactions to several β-lactam antibiotics. Haptenization of proteins is thought to occur by aminolysis of the β-lactam ring by lysine residues. All four types of hypersensitivity reactions to penicillins have been documented, and these results have been extensively reviewed (36).

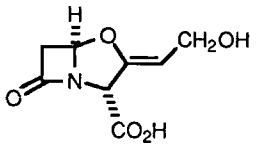


Three recent studies highlight some of the basic principles of drug hypersensitivity. Two groups have examined sera from patients diagnosed as allergic to penicillins (37, 38). Hapten

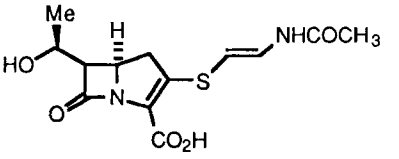
inhibition studies using various penicillins and cephalothin (3) as well as skin testing show that the immune system is able to discriminate between closely related haptens. Consequently, the specificity of an individual's immune response may allow a person who is allergic to one member of the β-lactams to be treated with another member of this class of antibiotics with impunity. This work also shows the heterogeneity of responses among individuals, highlighting the idiosyncrasy of drug reactions. Recent work with clavulanic acid (4) and MM22383 (5) illustrates the stability requirement for an immunogen (39). While 5 forms a single stable protein adduct, the clavulanic acid-protein product is unstable and breaks down rapidly to several products. MM22383 (5) is immunogenic in rabbits and produces a Type IV reaction in guinea pigs, whereas 4 induces only very mild responses. In addition, IgE antibodies to 5 were found in the serum of a worker exposed to the drug during its manufacture. These results support the hypothesis that not only must the hapten-protein conjugate be intrinsically immunogenic, but it must also exhibit a sufficiently long half-life to allow adequate stimulation of the immune system.



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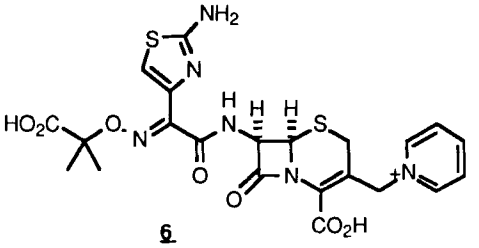


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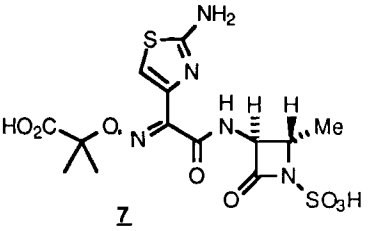


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The more recently developed β-lactams show early promise of greater clinical utility than their predecessors. For example, the third generation cephalosporin ceftazidime (6) and the monobactam aztreonam (7) are less immunogenic than other β-lactams in rabbits, and have little or no cross-reactivity with preformed antibodies to β-lactams (40-43). The specific reasons for this reduced immunogenicity are unclear. While the clinical experience with 7 has been encouraging, a 2% incidence of potentially immunologically mediated adverse reactions has been observed (41), and two anaphylactic reactions to this drug have recently been reported (44, 45). Whether the promise of improved clinical profile of 6 and 7 will be realized is still an open question.

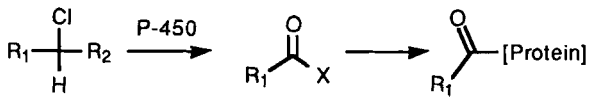


6



7

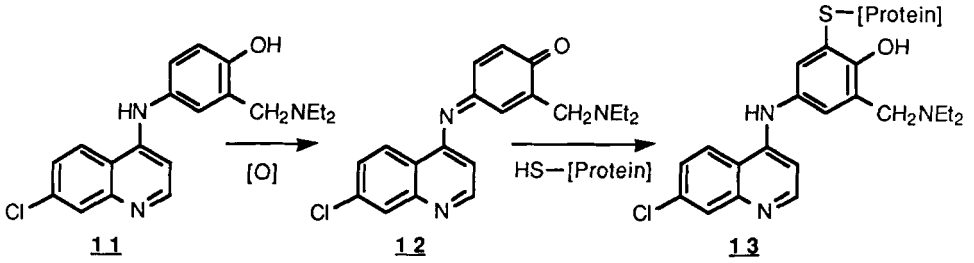
Halothanes - A growing body of evidence strongly suggests that acylation of liver proteins is responsible for the rare but potentially lethal occurrence of halothane-induced hepatitis (6). In addition to halothane (8), the related anesthetics enflurane (9) and isoflurane (10) have been implicated in this form of immune-mediated hepatotoxicity (46). It has been established that these compounds are oxidized in the liver by cytochrome P-450 to reactive trifluoroacetyl (TFA) metabolites which acylate liver proteins such as carboxylesterase (47). The neoantigens generated in this manner stimulate the production of IgG antibodies leading to a Type II reaction. Antibodies reactive with liver neoantigens have been found in serum of patients suffering from halothane-induced hepatitis (46-48). Inhibition of antibody binding to TFA-neoantigen with ε-*N*-TFA-lysine is weak, suggesting that the antibody combining site encompasses the hapten and part of the protein to which it is attached (49). These data highlight the difficulty of demonstrating an immune mediated etiology without first identifying the appropriate hapten-carrier conjugate.



	R1	R2
8	CF_3	Br
9	CF_3OCF_2	F
10	CF_3	$OCHF_2$

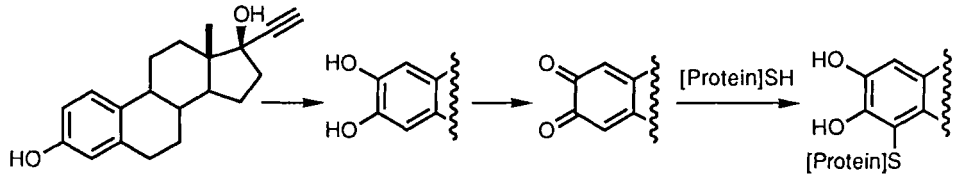
Interestingly, there is a positive correlation between the MHC haplotype Aw24-Bw52-DR2 and the severity of halothane hepatitis (50). As discussed above, the MHC glycoproteins are both a central controlling feature of the immune response and highly polymorphic. This observation may help explain the idiosyncratic nature of halothane and other hypersensitivity reactions and suggest that hypersensitivity reactions may remain unpredictable until all "sensitive" MHC haplotypes for a particular drug are identified and cataloged.

Quinone Precursors - Oxidation of aromatic rings is an important metabolic pathway by which xenobiotics may be activated prior to conjugation with proteins. For example, the antimalarial amodiaquine (11) can be oxidized by air or horseradish peroxidase to quinone imine 12, which then conjugates to protein by sulfhydryl Michael addition *in vitro* (51, 52). Since formation of 12 has never been observed *in vivo*, this immunogenic pathway remains hypothetical. However, antibodies to 11 have been detected in the sera of hypersensitive individuals in man and rat, and a reactive metabolite of 11 is produced by activated human polymorphonuclear leukocytes (PMN) *in vitro* (53-55). These antibodies are predominantly of the IgG isotype and generate a Type II reaction resulting in the destruction of neutrophils. While covalent binding of the drug or its metabolite to the affected cell types has been difficult to demonstrate, the antibodies isolated from hypersensitive patients are clearly specific and drug dependent.



11 12 13

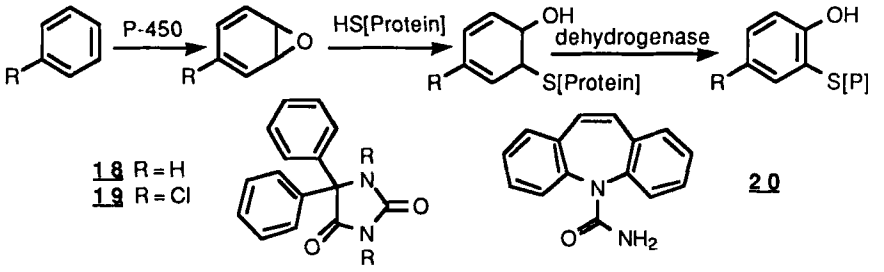
A related mode of conjugation has been proposed to explain the occurrence of thromboembolism caused by the oral contraceptive ethinyl estradiol (14). *In vivo*, 2-hydroxylation of the A ring (15) is usually followed by glucuronidation or sulfation (56), but *in vitro* oxidation with human liver microsomal preparations leads to extensive binding to hepatic proteins (23, 57). The inhibition of protein binding by ascorbate accompanied by accumulation of 15 implicates *o*-quinone 16 as the reactive metabolite responsible for protein conjugation. Although this reaction sequence has not been characterized *in vivo*, IgG antibodies to 14 have been found in women taking oral contraceptives (OC) but not in untreated women (58-61). Antibodies were found in 11 of 12 women taking OC (58), and women having undergone a thrombosis had the greatest amount of antibody in their serum. Circulating immune complexes depositing along blood vessel walls is indicative of a Type III hypersensitivity response.



14 15 16 17

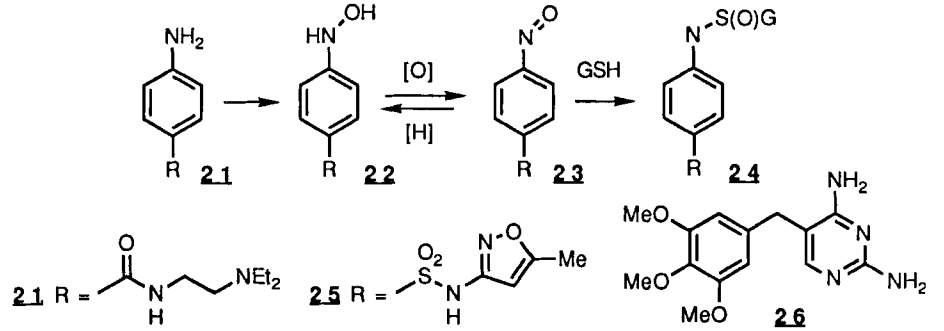
Hydantoins - Aromatic rings are oxidized to arene oxides by cytochrome P-450. Under normal circumstances, these arene oxides isomerize to phenols prior to glucuronidation or sulfation. In some cases, however, the reactive arene oxide is trapped by a protein nucleophile (probably RSH)

and becomes immunogenic. This mechanism of protein conjugation has been proposed to explain the occurrence of hypersensitivity to the anticonvulsants phenytoin (**18**) (62, 63) and carbamazepine (**20**) (64, 65). For example, **18** is metabolized to a reactive species by mouse liver microsomes which covalently attaches to soluble protein (66). Curiously, metabolites generated by murine microsomes are cytotoxic for mononuclear blood cells while the same product generated by human microsomes is not (66, 67). Phenytoin is also converted to *N,N*-dichloro derivative **19** by either myeloperoxidase or activated neutrophils *in vitro* (68). The mechanism by which this compound binds to neutrophils or serum protein is not known, but **19** has been implicated in the occurrence of lupus-like syndrome (10).



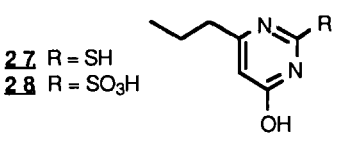
In humans, reactions characteristic of Type II, III and IV hypersensitivities have been described for **18**, and any one individual may manifest one or more types and in varying degrees (8). The most consistently described hypersensitivity appears to be of the Type IV class. This probably results from chemical derivatization of lymphocytes and bystander tissue (rather than soluble proteins) leading to direct cell destruction by cytotoxic lymphocytes and indirect cell destruction from DTH-cell-derived inflammatory mediators.

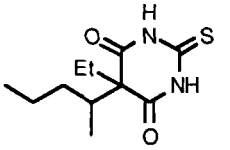
Anilines - A variety of anilines and heterocyclic aryl primary amines are known to cause hypersensitivity reactions through reactions of their metabolites (10). For example, procainamide (**21**) is metabolized first to the corresponding hydroxyl amine **22** and then to nitroso derivative **23** (69, 70). *In vitro,* hydroxyl amine **22** fails to react with glutathione (GSH) in the presence of antioxidants (ascorbic acid or NADPH) or if oxygen is rigorously excluded. Aryl nitroso **23**, on the other hand, rapidly reacts with glutathione, and the resulting sulfenamide (**24**) can be isolated by HPLC. Metabolites of **21** bind efficiently to albumin and histone under similar conditions, and are also toxic to leukocytes (71). Destruction of leukocytes could lead to a loss of normal immune regulation and covalent binding of active metabolites to cell surfaces could lead to autoimmune cytotoxic and DTH responses as described above for phenytoin. Tissue destruction resulting in the release of nuclear material may account for the appearance of anti-histone antibodies in procainamide hypersensitive patients (72, 73). Because of these antibodies, patients display predominantly a Type III hypersensitivity response. Additional clinical evidence suggests that hypersensitivity to aryl amines is a consequence of a "defect" in the usual N-acetylation metabolic pathway (74). Under normal conditions, a polymorphic enzyme converts primary aryl amines into non-toxic, acetamides (i.e. "fast acetylation"), but some people are "slow acetylators", leaving a significant concentration of aryl amine available for oxidation to the reactive nitroso metabolite. Subsequent protein conjugation apparently leads to hypersensitivity in these individuals.



Antibody binding inhibition studies with analogs of the antibacterial agents sulfamethoxazole (25) and trimethoprim (26) also support the conclusion that haptenization occurs through activation of the aryl amine moiety (1, 75, 76). Unlike procainamide (21), sulfamethoxazole (25) and trimethoprim (26) hypersensitive patients usually have IgE antibodies in their serum, indicative of a Type I hypersensitivity response (77-79). Even though 25 and 26 are usually administered in combination, the patients tested appeared to have antibodies to one or the other but not to both drugs. IgG and IgM antibodies to 25 and its metabolite has also been described in two patients with an immune mediated (Type II) thrombocytopenia (80).

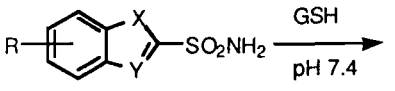
Aryl Sulfur Compounds - Heterocyclic aryl thiols and thiones such as propylthiouracil (27) are oxidized *in vitro* to sulfates by activated lymphocytes or myeloperoxidase (10, 81). Sulfate 28 adds to sulfhydryl-containing peptides such as glutathione by nucleophilic aromatic substitution, and may conjugate to proteins *in vivo* in a similar manner. Stimulation of lupus-like responses in both cats (82, 83) and humans (84, 85) as well as anti-neutrophil antibody production and agranulosis (86, 87) have been attributed to 27. The pathology is similar to that described above for procainamide (21), and may be induced by a similar mechanism. For the barbiturate thiopentone (29), on the other hand, specific IgE antibodies have been found in hypersensitive patients (88-90). In two patients who experienced an anaphylactic episode to 29, significant antibody cross-reactivity with other classes of neuromuscular blocking drugs (NMBDs) was observerd (88). Consequently, prior exposure to NMBDs may exacerbate hypersensitivity responses to 29.





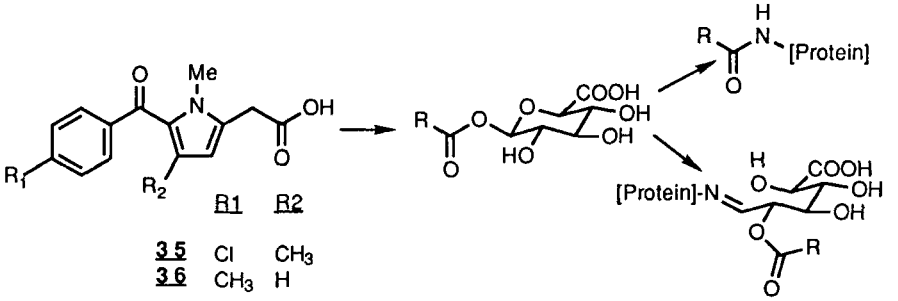
29

The structurally related benzothiazole-2-sulfonamide carbonic anhydrase inhibitor 30 and indole 31 are potent contact sensitizers in test animals (91, 92). Other analogs such as benzofurans 32 and indole 33 are less immunogenic, and benzothiophene 34 fails to provoke an allergic response under these conditions (92, 93). For these compounds, *in vitro* reactivity with reduced glutathione (GSH) correlates qualitatively with a Type IV dermal sensitization of guinea pigs.



	X	Y	R
30	S	N	6-OCOC(CH3)3
31	N	CH	5-OH
32	O	CH	5-OH
33	O	CH	6-OH
34	S	CH	6-OH

Aryl Acetic Acids - Immunological reactions to the non-steroidal anti-inflammatory drugs (NSAIDs) zomepirac (35) and tolmetin (36) have been reported (94-97). 1-*O*-Glucuronic esters of 35 and 36 covalently bind to plasma proteins *in vivo* (98-100). Rearrangement of the initially formed 35 1-*O*-glucuronide to the 2-*O* isomer followed by C-1 Schiff base formation with lysine residues also occurs *in vitro*, but no immunological effects have yet been attributed to this metabolite (101).



While **35** and **36** produce symptoms of a classical Type I response in hypersensitive individuals, the presence of specific IgE antibodies and positive skin tests have been difficult to demonstrate (96, 102, 103). However, IgG and IgM antibodies reacting to **35** and **36** have been found in patients' serum, and antibodies reactive to the patients' red cells (Type II response) have been documented (104, 105). Consequently, it appears that these drugs cause two different types of hypersensitivity reactions. The first has been termed an "anaphylactoid" response to distinguish it from an authentic IgE mediated "anaphylaxis". The "anaphylactoid" response is likely due to the suppression of cyclooxygenase resulting in mast cell instability and a spontaneous release of vasoactive amines (106, 107). The second hypersensitivity response to **35** and **36** results in hemolytic anemias and disseminated intravascular coagulation, and is mediated by specific antibodies.

Conclusion - It is often difficult to convincingly demonstrate an immune based etiology for adverse drug reactions and, consequently, many adverse effects of therapeutic agents have been classified as immune-mediated based purely on clinical symptoms. Predicting adverse reactions of novel therapeutic agents has been even more difficult. Immune-based reactions can be anticipated in a handful of well-defined drug classes, however consistent prediction of hypersensitivity reactions as a branch of structure/toxicity is still beyond our current capabilities for the vast majority of drugs. The dependence of the immune response on the highly polymorphic MHC gene system is one element which confounds our ability to predict hypersensitivity reactions and genetic polymorphisms which regulate IgE levels and basophil degranulation impose additional complexities. The fact that metabolism of drugs to reactive species often precedes protein conjugation and immunogen formation adds further complications. Consequently, until we understand the individual's MHC gene profile, the nature of the immunogen, which MHC glycoprotein(s) effectively present the immunogen, and the genetic disposition relating to IgE levels and basophil degranulation, drug hypersensitivity responses are likely to remain largely idiosyncratic. Nevertheless, defining reaction mechanisms for drugs and their metabolites serves as an important guide to predicting structure/toxicity relationships leading to immune-mediated drug hypersensitivity for novel compounds.

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Chapter 20. Cholecystokinin Agonists and Antagonists

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Introduction - Cholecystokinin (CCK) is a polypeptide hormone found in the periphery that plays a major role in gut function, in the digestive process and in the control of feeding behaviors (1-3). CCK also occurs in brain where it acts as a neurotransmitter and neuromodulator (4). In certain brain regions, CCK co-exists with other neurotransmitters such as dopamine (DA), Met-enkephalin and GABA, and appears to modulate select actions of these agents. Numerous CNS behaviors have been reported for CCK, when given either systemically or intraventricularly, including analgesic, anorectic, anticonvulsant, sedative and neuroleptic-like actions. The literature is extensive and the reader is referred to several excellent reviews for more comprehensive treatments (5-9). Over the last 10-15 years, CCK research has focused on establishing the functional significance of this peptide in the CNS (4,5), the importance of CCK in the regulation of feeding behaviors (2,3), the respective roles of CCK receptor subtypes in the periphery and CNS (6), and the potential for CCK-based compounds as sources of novel therapies for the treatment of a variety of gastrointestinal (10,11) and neuropsychiatric disorders (4,5). Until recently, progress has been hampered by the lack of selective agents for CCK receptor subtypes, the chemical and enzymatic lability of peptide probes, and the inability of such agents to cross the blood-brain barrier. The development of several highly potent, selective CCK agonists and antagonists has now provided powerful pharmacological tools for addressing the above issues. These agents may lead to novel therapy for the treatment of disorders in which CCK has been implicated including anxiety (12), cognitive dysfunction (13), gastrointestinal disturbances (10), obesity (14), pancreatitis (11), panic (15), pain (16), schizophrenia (17), and possibly, substance abuse (18). This chapter will focus on both non-peptide and peptide CCK agonists and antagonists which exhibit high selectivity for CCK receptor subtypes or possess enhanced chemical and enzymatic stability or superior physicochemical properties over the parent peptide. The use of these agents as biological probes and their potential as therapeutic agents will be addressed.

Background - CCK exists in multiple biologically active forms (CCK-58, CCK-39, CCK-33, CCK-8 and CCK-4), with CCK-33, CCK-8 and CCK-4 predominating in the periphery (8). The primary form of CCK in brain is the C-terminal octapeptide, CCK-8 (Figure). Thus far, two major CCK receptor subtypes have been characterized. CCK-A ("alimentary") receptors predominate in peripheral target organs such as pancreas, gallbladder and ileum, and on vagal afferent fibers, where they mediate pancreatic enzyme secretion, gallbladder and ileum contractions, and food intake, respectively (1-3). Type-A receptors also have been detected, albeit in low concentrations, in discrete brain regions (19,20). These receptors appear to modulate select actions of DA (21) and may centrally regulate feeding behaviors in some species (3). CCK-B ("brain") receptors exist in numerous brain regions including cerebral cortex, hippocampus and limbic structures (4-6). CCK-B-like receptors also have been reported in the periphery and may be similar or identical to peripheral gastrin receptors (22-24). The functional significance of brain CCK receptors has been elusive, although recent evidence suggests a role in modulation of anxiety and pain (25) and, perhaps, regulation of certain feeding behaviors (26). CCK receptor subtypes can be distinguished by their relative affinities for various CCK fragments, with CCK-A receptors demonstrating high affinity for CCK-8 and low affinity for the desulfated form, CCK-8(DS), and for CCK-4, the C-terminal tetrapeptide fragment (Figure), also known as tetragastrin (27). In contrast, CCK-B receptors are less discriminating and show comparable affinity for these fragments as well as for gastrin (28).

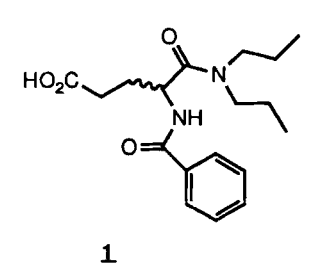
1 2 3 4 5 6 7 8
Asp - Tyr(SO_3H) - Met - Gly - **Trp - Met - Asp - Phe - NH_2**

Figure: Amino acid sequence for CCK-8 and CCK-4 (in bold).

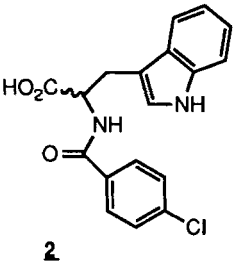
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ANTAGONISTS

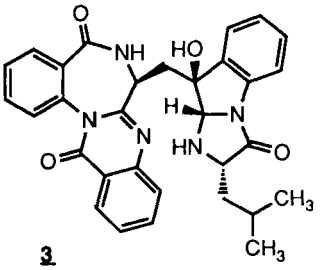
Benzodiazepines - Prior to the recent advances in the CCK field only a few relatively weak and non-selective antagonists such as proglumide (1), benzotript (2), and dibutyryl cyclic GMP were known (29-31). None of these demonstrated any receptor differentiation and possessed IC_{50}'s > 500 μM. A major advance was the discovery of asperlicin (3) (IC_{50} = 1μM), a fermentation product from *Aspergillus alliaceus*, as a non-peptide CCK-A antagonist (32). Synthetic and biosynthetic approaches provided a variety of analogs, but none possessed oral bioavailability (33-35). Independent and concurrent research demonstrated the ability of simple benzodiazepines (e.g. diazepam) and β-carbolines to antagonize CCK actions (36-38). Tifluadom (4) was found to inhibit CCK-A receptor binding (IC_{50} = 47 nM) and function (39). The recognition of the underlying 1,4-benzodiazepine structure of asperlicin led to devazepide (MK-329, L-364,718, 5), a potent (IC_{50} = 0.08 nM), orally active and selective CCK-A antagonist (40-42) and L-365,260 (6) a potent (IC_{50} = 2 nM) and selective CCK-B antagonist (43,44). It is noteworthy that the *S* enantiomer of 6 demonstrated selectivity for the CCK-A receptor (43). Radioligands of both 5 and 6 have been prepared (45) and studies with 5, 6 and [^{125}I-BH]-CCK-8 have allowed the differentiation of CCK-A and CCK-B receptors within the brain and spinal cord in a number of species (46). Both 5 and 6 have been studied in various analgesic models and have been found to potentiate opiate-induced analgesia and to prevent tolerance to morphine (47-49). Based on the selectivity of these two compounds, it was found that blockade of CCK-B receptors induces an antinociceptive effect in primates (49). Devazepide was found to potently inhibit pancreatic function, gallbladder contraction and gastric function in a number of species (50-53). Devazepide inhibited the anorectic action of CCK-8, caerulein, or fenfluramine in animal models and, in some paradigms, increased food intake when administered alone (54-59). In human subjects, 5 (10 mg, p.o.) inhibited CCK-8 or meal-stimulated gall bladder contractions and pancreatic exocrine function while gastric emptying rates remained unchanged (60,61). Plasma CCK levels were notably higher (13.8 vs 2.3 pM) in devazepide versus placebo treated subjects. Doses higher than 10 mg were reported to induce gastrointestinal side-effects (61).



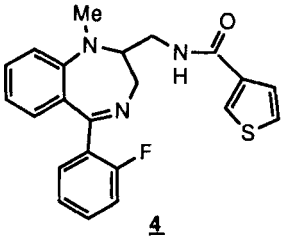
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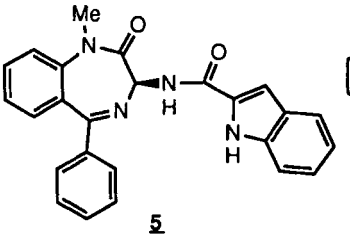
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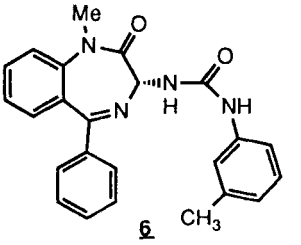
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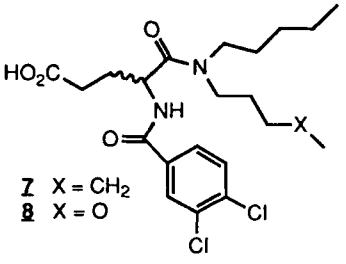
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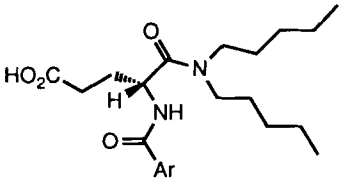
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Proglumide and Benzotript Analogs - Lorglumide (CR 1409, 7) was introduced as an advance over the prototypical antagonist proglumide (62-64), and has now been superseded by loxiglumide (CR 1505, 8) in clinical trials (65). A number of research groups have reported that the *R* enantiomer in this series is the more potent agent for CCK antagonism and compounds 9-11 have been reported as pure enantiomers with increased potency and selectivity compared to 7 and 8 (66,67). CCK-A antagonist models have been proposed based on the similarity of compounds 5 and 9 and related

analogs (68). The *in vitro* pharmacology of loxiglumide **8** has been reported extensively and its potency confirmed in *in vivo* models (69,70). Loxiglumide inhibited CCK-8 induced gallbladder and pyloric contractions, pancreatic secretions, stomach emptying, and satiety in various species (71). Loxiglumide also ameliorated acute pancreatitis induced in rodents by caerulein, sodium taurocholate, or choline deficient diet (72,73). Pharmacokinetic studies in man have indicated a biphasic elimination rate ($t_{1/2}$ = 33 min, 6.3 h) and good oral bioavailability (74). Loxiglumide (10 mg/kg/h, i.v.) inhibited both meal-stimulated and CCK-8-stimulated pancreatic and biliary secretions in normal humans (75-77). At a dose of 2.5 - 5 mg/kg/h, i.v., **8** significantly inhibited postprandial gallbladder contraction induced by a test meal (78) or stimulated by either caerulein or bombesin (79-83). Similar doses were shown to accelerate postprandial gastric emptying and gastric emptying induced by i.v. CCK-8 (84,85). In one study pretreatment with loxiglumide increased CCK plasma levels induced by bombesin or a test meal (86). In patients with moderate to severe exocrine pancreatic insufficiency **8** (10 mg/kg/h, iv) was effective in reducing meal-stimulated pancreatic enzyme output, thereby demonstrating that endogenous CCK is involved with regulation of pancreatic secretion even in patients with deficient pancreatic function (87).

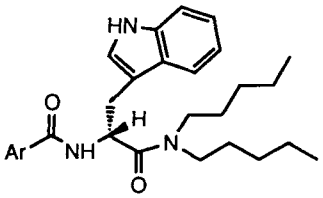


7 X = CH_2
8 X = O



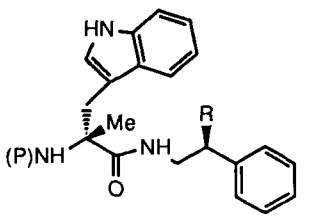
9 Ar = 2-indolyl
10 Ar = 3-quinolyl
11 Ar = m-methoxyphenylamido

Improvements upon the benzotript structure have been achieved by utilizing an antagonist model similar to **5** and **9**. Compounds **12** (IC_{50} = 23 nM) and **13** (IC_{50} = 21 nM) are representative of these structures which have marked parallels to the proglumide-like compounds and are selective for CCK-A receptors (88).



12 Ar = 3-quinolinyl
13 Ar = 4,8-dihydroxy-2-quinolinyl

Peptide-Based Antagonists - Synthetic modifications of the CCK peptide also have produced a number of important findings. Compound **14** is a dipeptoid discovered from a deletion approach (89). Although these dipeptoids were weak (IC_{50} ~1-10 μM), further research provided the novel dipeptoid CI-988 (PD-134308, **15**) with enhanced potency (IC_{50} = 1.7 nM) and selectivity for the CCK-B receptor (90). CI-988 possessed >1000 fold selectivity for CCK-B receptors, inhibited gastric acid secretion when administered i.v. in the Ghosh-Schild assay, and demonstrated significant oral activity (0.0001-10 mg/kg) in mice in the black-white box anxiolytic paradigm (12). CI-988 has been further characterized in the latter assay and shown to be nonsedative, free of withdrawal anxiogenesis following chronic treatment, and effective in preventing withdrawal anxiogenesis following diazepam, nicotine or alcohol treatment (18). CI-988 was effective in other anxiolytic assays including the rat social interaction test and the marmoset "human threat" test. Moreover, CI-988 was shown to potentiate morphine analgesia (16).



14 R = H, (P) = 1-Adoc
15 R = $NHCOCH_2CH_2CO_2H$, (P) = 2-Adoc

Working from the parent CCK peptide, a number of groups have reported pharmacologically interesting antagonists. Deletion of the carboxy terminal Phe from CCK produces an antagonist **16** (IC_{50} = 3 μM) in guinea pig pancreatic acini which was subsequently shown to be a partial agonist in mouse and rat (91,92). An extension of this design concept was JMV-170 (**17**), the phenethylamide (93,94), and JMV-180 (**18**), its corresponding phenethyl ester (95,96). Compound **18** proved interesting since it possessed full agonist properties at the high affinity CCK-A receptor in rat

pancreatic acini while possessing antagonism at the low affinity receptors (97). Simple substitution to yield **19** provided a more potent CCK-A antagonist (IC_{50} = 30 nM) (98). Another interesting pharmacological profile is that of **20**, which is a potent agonist at CCK-A receptors (IC_{50} = 5 nM) and an apparent antagonist (IC_{50} = 0.7 nM) at the CCK-B receptor (99). The hydrazide **21** also presented bifurcated pharmacology demonstrating antagonism at guinea pig CCK-A receptors and agonism at CCK-B receptors (100). Compound **22** (IC_{50} = 230 nM) was more potent than compound **16** at inhibiting CCK-A receptor binding and function. Extension of **22** to **23** increased CCK-B binding affinity without altering the CCK-A antagonist activity (101).

P - Tyr(SO_3H) - Met - Gly - X - Met - Asp - R

16 P = Cbz, X = Trp, R = NH_2

17 P = Boc, X = Trp, R = $NH(CH_2)_2$ - phenyl

18 P = Boc, X = Trp, R = $O(CH_2)_2$ - phenyl

19 P = Boc, X = D - Trp, R = $O(CH_2)_2$ - phenyl

21 Boc - Trp - Leu - Asp - Phe - $NHNH_2$

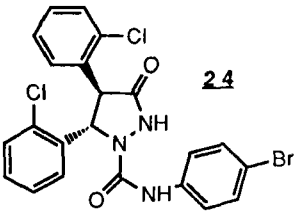
P - Trp - Orn(Cbz) - Asp - NH_2

22 P = Boc

23 P = Boc - Tyr(SO_3H) - Nle - Gly

20 Boc - Try(SO_3H) - NleΨ[$COCH_2$]Gly - Trp - Nle - Asp - Phe - NH_2

Miscellaneous Antagonists - Pyrazolidinones, of which LY-262864 (racemic **24**) is a representative example, demonstrate selectivity for gastrin and CCK-B receptors (IC_{50} = 6 nM) over CCK-A receptors and are competitive antagonists (102). Other heterocycles similar to **5** are under study (103). Antibodies to CCK represent another antagonist approach which has been studied in conjunction with food intake in livestock (104).

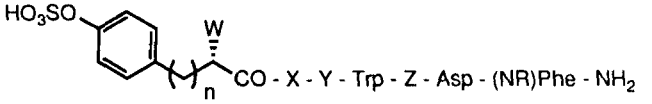


24

Therapeutic Potential - A number of possible utilities have been mentioned for CCK antagonists including treatments for pancreatitis, insulinoma, gastroparesis, irritable bowel disease, pancreatic and gastric carcinomas and as adjuncts to analgesics (10,105). The viability of these potential uses awaits clinical trials in man. Evidence from animal models indicates that CCK antagonists may have a role in the treatment of acute pancreatitis (11) and in some forms of pancreatic or gastric cancers (106-108). CCK has been linked to the GABAergic, dopaminergic and cholinergic systems in the CNS (109). CNS therapies have centered on anxiolytics (12,18) and disorders related to dopaminergic dysfunction such as Parkinson's disease and schizophrenia (110). Other possibilities include the treatment for addiction to substances of abuse such as nicotine and alcohol (18).

AGONISTS

Nonselective Analogs - Numerous SAR studies on CCK-8 have afforded peptide analogs with improved chemical and/or enzymatic stability for use as pharmacological tools or as potential anorectic agents. Targeted positions have included the chemically labile Met and Tyr-sulfate moieties as well as the N-terminal amino function and the Met-Gly, Gly-Trp, and Asp-Phe bonds which are most susceptible to proteolytic degradation (111). A detailed SAR investigation on CCK-8 provided several potent, protease resistant, long acting anorectics (112). Most notable were compounds **25** and **26**, exhibiting >100 and 19-fold enhancement of stability (kidney homogenates) with anorectic potencies equivalent to and 10-100-fold greater than CCK-8, respectively. A strong correlation was established between potency on contraction of gallbladder (EC_{50}) and anorectic efficacy (% inhibition) for this series (Spearman's r = -0.81, $p < 0.001$). Recently, a related analog, FPL 14294 (**27**), was disclosed (113). FPL 14294 was over 50-200 times more potent than CCK-8 when administered to rats by either the i.p. or nasal routes.



25 n = 1, W = H, X = Z = Met, Y = D-Ala, R = Me

26 n = 1, W = H, X = Z = Met, Y = Gly, R = Me

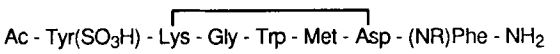
27 n = 0, W = H, X = Z = Met, Y =Gly, R = Me

28 n = 1, W = Boc - NH, X = Z = Nle, Y = Gly, R = H

29 n = 1, W = H, X = Nle, Y = Gly, Z = t-3-propylPro, R = Me

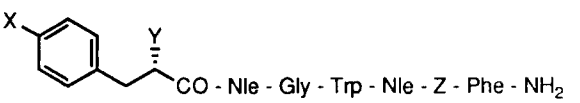
Substitution of Met with the bioisosteric Nle in Boc-CCK-7 gave **28** having improved stability and similar potency to CCK-8 on both peripheral organs (pancreas, ileum, gallbladder) and in the CNS (hypomotility), but with somewhat reduced anorectic efficacy in rats (114,115). A related analog, A-68552 (**29**), containing constrained amino acids *trans*-3-n-propylproline and (N-Me)Phe, was reported to have comparable affinities at brain and pancreatic receptors, full agonist activity in amylase release, with enhanced anorectic potency and prolonged duration of action over CCK-8 (116).

Introduction of conformational constraints into CCK-7 by means of side-chain to side-chain cyclization produced moderately potent cyclic heptapeptide analogs. Among the 20 analogs prepared, only the replacement of Met^2 with Lys and subsequent cyclization of the ε-amino function to the side-chain carboxyl of Asp^6 produced active compounds (117). Analog **30** (R=H) exhibited modest affinity for type-A (IC_{50} = 620 nM) and type-B (3600 nM) receptors with 30-fold weaker potency (ED_{50} = 210 μg/kg, i.p.) than CCK-8 in suppressing food intake in rats. Interestingly, the N-methyl analog of **30** (R = Me) had one-eighth the affinity for pancreatic receptors, but was almost twice as potent in suppressing food intake. Anorectic actions of both compounds were blocked by the selective CCK-A antagonist, MK-329. The relatively modest affinity and potency of **30** is likely due to the absence of the free carboxyl function at Asp^6, which has been shown previously to be required for high affinity and efficacy in CCK-A receptor mediated actions (118). Additional amino acid substitutions in **30** at Gly^3 or Met^5 yielded inactive analogs, as did attempts to cyclize in other modes (117).



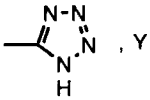
30

Three biologically acceptable replacements for the acid-labile tyrosine sulfate moiety of CCK-8 have been described. Incorporation of Ac[L-Phe(CH_2SO_3H)] into **28** gave analog **31** having high affinity (K_i ~10^{-9} M) for both CCK receptors with full agonist activity in pancreatic amylase secretion and in contraction of ileum (119,120). Similarly, incorporation of Ac[L-Phe] with CO_2H, CH_2CO_2H, CF_2CO_2H, tetrazolyl or methyltetrazolyl at the 4-position afforded CCK-7 analogs which demonstrated acceptability of both CH_2CO_2H and tetrazolyl as chemically stable alternatives for the sulfate ester moiety (121). Both **32** and **33** had favorable affinity for CCK-A receptors (IC_{50} ~3 nM) with comparable potency to the parent in suppressing food intake in rats. Moreover, the corresponding desamino analogs of **32** and **33** (Y = H) were an order of magnitude less potent in binding to pancreatic receptors, but proved of nearly equal potency to their parents as anorectics.



31 X = CH_2SO_3H, Y = Boc - NH, Z = Asp

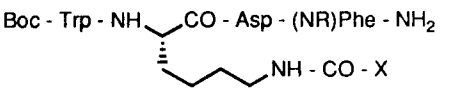
32 X = CH_2CO_2H, Y = Ac - NH, Z = Asp

33 X =  , Y = Ac - NH, Z = Asp

34 X = OSO_3H, Y = H, Z = (N-Me)Asp

Type-A Selective Analogs - Although several classes of highly potent and selective CCK-A antagonists are available, only very recently have selective agonists been disclosed. Substitution of Asp^6 with (N-Me)Asp in desamino [Nle^2, Nle^5]CCK-7 provided A-71378 (**34**). Reported activities for A-71378 include high affinity (IC_{50} = 0.42 nM) and selectivity (700-fold) for pancreatic receptors and full agonist activity in stimulation of amylase release and ileal contractions (122). Consistent with it's relatively poor affinity for brain receptors, A-71378 was weak in mobilization of intracellular calcium from NCI-H345 cells which express CCK-B/gastrin receptors.

Two novel, closely related series of A selective CCK tetrapeptides, represented by **35** and **36** (R = H or Me), were developed by modification of CCK-4 analog **37** (R = H) containing Lys(ε-Cbz) in place of Met^2 (123,124). Although **37** showed only modest affinity for CCK-A receptors on pancreas (IC_{50} = 510 nM), it exhibited an unexpected 10-fold selectivity for the



35 X = 2- methylphenylamino

36 X = t -2-(4-hydroxyphenyl)ethenyl

37 X = benzyloxy

A subtype, opposite to that observed for other CCK-4 analogs. SAR studies revealed that type-A selectivity and affinity were critically dependent on the nature of the substituent attached to the ε-amino function and on the distance of the substituent from the peptide backbone (125). The most potent member of the series, A-71623 (**35**, R = Me), had high affinity (IC_{50} = 4 nM) and over 1000-fold selectivity for CCK-A receptors, full agonist activity in release of pancreatic amylase, gallbladder and ileum contractions, and high potency in suppression of food intake in rats (ED_{50} = 3 nmol/kg, i.p.) and monkeys (10 nmol/kg, i.m.). Significantly, when rats were given A-71623 once daily for a period of 10 days, animals gained less weight relative to controls and did not develop tolerance to its anorectic effects as has been reported for CCK-8 (126).

Type-B Selective Analogs - Incorporation of (N-Me)Nle residues for Met in CCK-8 and CCK-5 have lead to highly potent analogs **38**, **39**, and **40**, possessing 5100, 9100 and >3000-fold selectivity, respectively, for CCK-B receptor sites (127). Both affinity and selectivity were dependent on the number and positions of the (N-Me)Nle residues, as well as the degree of sulfation, with desulfated analogs generally more selective (**40** is an exception). NMR studies suggest that cis/trans isomerism about the (N-Me)Nle residue may be related to the high selectivity. Potency and selectivity of these analogs can be further enhanced by replacing (N-Me)Nle^3 with either *threo*-L- or D-β-MePhe (128). Two similar CCK-B selective analogs, namely A-72962 (**41**) and BC 264 (**42**), also have been described. A-72962 exhibited high affinity (IC_{50}=0.19 nM) and 579-fold selectivity for cortical receptors, was weak in amylase and ileal contraction assays, and behaved as an agonist in stimulating calcium mobilization in NCI-H345 cells (122). The presence of the retro-inverso amide bond between Nle-Gly in BC 264 served to enhance the selectivity for type-B sites (K_i = 0.15 nM, 520-fold) and to further protect the peptide from degradation (129). BC 264 proved weak in CCK-A mediated assays but was shown to be a potent agonist in type-B CNS actions including stimulation of rat CA hippocampal neuron firing and potentiation of dopamine-induced hypolocomotion when injected directly into the VTA of rats (130). BC 264 has been used to characterize CCK-B functional actions in the CNS (131,132). A tritiated form has been reported (133) and utilized for blood-brain barrier studies (134).

X - Trp - Y - Asp - Phe - NH_2

38 X = Gly, Y = (N-Me)Nle

39 X = Asp - Tyr - (N-Me)Nle - Gly, Y = (N-Me)Nle

40 X = (N-SO_3H)Asp - Tyr(SO_3H) - (N-Me)Nle - Gly, Y = Nle

41 X = Desamino -Tyr(SO_3H) - Nle - Gly, Y = (N-Me)Leu

42 X = Boc - Tyr(SO_3H) - *g*Nle - *m*Gly, Y = (N-Me)Nle

Boc - X - Tyr(SO_3H) - Nle - D-Lys - Trp - Nle - Asp - Phe - NH_2

43

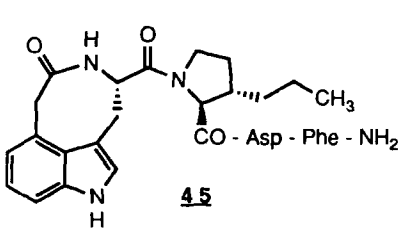
X - Lys - Gly - Trp - Lys - Asp - Phe - NH_2
|
OC - CH_2 - CH_2 - CO

44

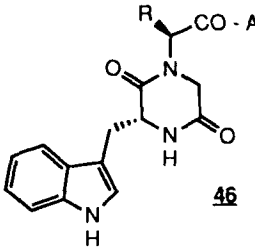
A novel series of cyclic peptides of general structure **43** (X = Asp, D-Asp, Glu, D-Glu, or γ-D-Glu) was prepared based on a conformational model of CCK-8 derived from NMR studies (135,136). The incorporation of D-Lys in place of Gly was intended to stabilize a β-turn around the sequence Asp-Tyr-Met-Gly in CCK-8. Two of the most potent analogs were obtained when X= D-Asp or γ-D-Glu, with the latter having greater potency and selectivity (K_i = 0.6 nM, 4464-fold). Both analogs were expectedly weak in CCK-A functional assays, neither being capable of inducing contractions in guinea pig ileum. To date, the only report of CCK-B functional activity for this series is for BC 197, **43** (X= D-Asp), which behaves as an antagonist of CCK-induced self-stimulation behavior in rats (137). A second series of cyclic analogs also was prepared based on conformational considerations to yield potent CCK-B selective ligands (138,139). JMV 310 [**44**, X = Ac-Tyr(SO_3H)], bound with high affinity to cortical receptors (IC_{50} = 1.3 nM) and was over 10,000-fold selective. The corresponding desulfated analog was one-half as potent but of comparable selectivity. Hexapeptide analogs **44** (X = Ac or H) exhibited comparable potency and selectivity to Boc-[Leu^2]CCK-4. CCK-B functional activity has yet to be reported for this series.

Finally, several novel conformationally restricted CCK tetrapeptides have been described (140,141). Since it is well-known that CCK-4 contains all the necessary elements for binding and activation of CCK-B receptors, and has moderate selectivity for brain over peripheral receptors, a series was developed by systematically replacing each of the amino acids in CCK-4 with a variety of natural, unnatural, conformationally constrained and dehydroamino acids (140). The most potent analog, A-63387 (**45**), bound to cortical receptors with an IC_{50} = 0.7 nM and type-B selectivity of 9000-fold. A-63387 also demonstrated high affinity for gastrin receptors and stimulated calcium

mobilization in NCI-H345 cells. A recent report of selective, diketopiperazine-containing analogs (46) also comprised part of this series (142).



45



46 R = n-butyl or iso-butyl

Therapeutic Potential - The indications for CCK agonists appear more restricted than for antagonists, with nonselective and CCK-A agonists having potential as anorectic agents. Although compounds with enhanced potency, duration of action and efficacy upon repeated administration have been reported, several issues still remain regarding adverse side effect potential for CCK-based agents (14). CCK-A selective agonists may offer an advantage in this regard, since effects due to activation of CCK-B/gastrin receptors may be avoided. Activation of CCK-A receptors on pancreas and/or gallbladder remains a potential concern. Since CCK actions are highly species dependent, the utility of these new analogs awaits hypothesis testing in man. Furthermore, all reported agents are peptidic and lack oral activity. Thus, drug delivery via alternative routes (e.g. nasal, sublingual, inhalation) would be necessary, thereby limiting commercial potential. Therapeutic indications for CCK-B agonists are less encouraging in view of their cross reactivity with gastrin receptors and the production of anxiogenic-like effects reported for CCK-4 in man (15). Should any of the above CCK-B agonists demonstrate selectivity between gastrin and CCK-B receptors, they will aid in establishing which receptor is responsible for the observed anxiogenic effects. Otherwise, these compounds may serve as tools to identify and characterize additional CCK-B receptor subtypes in both peripheral and CNS tissues or provide conformationally restricted ligands as models of possible bioactive conformations upon which to base design of peptidomimetics.

CONCLUSIONS

The recent availability of receptor selective CCK agonists and antagonists has had a major impact on elucidating the functional significance of CCK in the periphery and in the CNS. However, much remains to be discovered. Ongoing clinical trials with CCK antagonists will ultimately determine the therapeutic potential of these novel agents and, perhaps, will uncover unanticipated utilities. In addition to serving as pharmacological tools for medical research, CCK selective agonists and antagonists will provide the medicinal chemist important information regarding structural and conformational requirements for binding and activation of CCK receptors, which will lead ultimately to new classes of non-peptide antagonists and, possibly, orally-effective peptidomimetic agonists.

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Chapter 21. Recent Advances in Bone Metabolism and Osteoporosis Research

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Introduction - Although the term osteoporosis (OP) is frequently used in reference to the process of abnormally accelerated bone loss (osteopenia), particularly that associated with the onset of the menopause, it is, in fact, a heterogeneous disorder clinically defined as a predisposition to bone fracture. It is the pathological end result of multiple causes and risk factors which include peak bone mass prior to the onset of aging-related bone loss and, in women, its exacerbation by the loss of ovarian hormonal support. It is currently estimated that as many as 20 million Americans suffer from OP, resulting in 1.2 million fractures yearly (1).

In the past several years there has been an exponential growth in our fundamental knowledge of bone metabolism and how defects in this system are reflected in disorders such as OP, Paget's disease, and osteomalacia. This chapter will provide an overview of some of the new developments in bone research at both the basic and clinical level (2).

BONE METABOLISM - MECHANISTIC INSIGHT

Growth Factors - Although the existence of specific factors responsible for the initiation and induction of bone growth was proposed over 25 years ago by the pioneering work of Urist (3), it has only been in the last few years that the possible identity of these factors has been reported. Through the use of classical analytical protein chemistry techniques and with the help of recent biotechnology advances it has been shown that a family of locally produced factors, variously designated as bone morphogenetic proteins (BMP), skeletal growth factors, osteins or osteogenic proteins, may be responsible for bone remodeling (4-6). There is general agreement that the primary endogenous factor is one or more of a group of proteins that are part of the transforming growth factor β (TGFβ) gene superfamily (5). Although various monomeric subunits (15-20 kD) of these proteins have been reported to be responsible for bone formation, it is likely, from experiences with other TGFβ related proteins, that the endogenous effector exists as a homo- or hetero-dimer (approx. 25-30 kD) (4, 6). While various claims on the utility of individual subunits of this family of BMP's have been made for local repair of bone defects, their ultimate role in bone remodeling and their utility for treatment of OP remain to be determined.

A large number of growth factors other than the BMP's have been identified. These factors may act in concert to direct the complex process of bone cell differentiation and function (7-9). This group includes TGFβ which appears to subserve a variety of roles including stimulation of growth of osteoblast progenitors, facilitation of osteoblast responses to other growth factors and inhibition of osteoclast formation and function. Subcutaneous administration of TGFβ to rats increased bone formation (10). Insulin-like growth factor-1, whose levels are normally regulated by growth hormone, is also a bone cell mitogen as well as a stimulus for collagen synthesis (11). Other factors that have been identified in bone include fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, insulin-like growth factor-2 and TGFα (12).

Cytokines - Cytokines have been implicated in the differentiation, mineral homeostasis and function of bone cells (9, 13, 14). Interleukin 4 (IL-4), a T cell derived stimulator of B cells, has been shown to inhibit bone resorption stimulated by a variety of agents (IL-1, PTH, 1,25 $(OH)_2$ D_3 and PGE_2) in a dose dependent manner (15). The specificity of this effect was demonstrated by blocking it with murine anti-IL-4 monoclonal antibody. This work suggests a role for the T cell in remodelling. In contrast, IL-6 has been shown to increase the transformation of cells from long term human marrow cultures into multinucleated cells which resemble the abnormal osteoclasts seen in Paget's disease (16).

Non-Collagenous Bone Proteins - Although type I collagen is the major organic constituent of bone, non-collagenous bone proteins account for 10% of the organic phase (17). Bone glycoproteins have been found to contain calcium and collagen binding sites [thrombospondin

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(18), osteonectin (19)] and to have an affinity for hydroxyapatite [osteonectin, osteopontin (20), bone sialoprotein (21), osteocalcin (22)]. As osteonectin binds to both hydroxyapatite and collagen, a role in binding mineral to organic matrix has been proposed (19). Hydroxyapatite binding has been associated with protein regions densely populated with carboxylated amino acids [bone sialoprotein contains ten consecutive glutamic acids (21) and osteopontin contains nine consecutive aspartic acids (20)]. Osteocalcin's hydroxyapatite binding has been associated with its three γ-carboxyglutamic acid residues (22). The Arg-Gly-Asp (RGD) sequence, which mediates ligand binding to cell surface integrins (23), is present in several non-collagenous bone proteins [thrombospondin, bone sialoprotein, osteopontin, fibronectin (24)]. RGD containing bone proteins and synthetic peptides have been shown to mediate osteoclast adhesion to inert surfaces (25, 26). In addition, the attachment to, or resorption of bone by osteoclasts has been disrupted by the presence of antibodies to the integrin receptor, or by the presence of synthetic peptides or bone proteins containing the RGD sequence (25, 26). Echistatin, an RGD containing venom protein, inhibited bone resorption by rat osteoclasts with an IC_{50} value of 0.1nM and was shown to bind to integrin receptors (27).

Bone Cell Estrogen Receptor - Estrogen deficiency, such as that occurring with the menopause, is associated with an acceleration in bone loss which is, in turn, a predisposing factor for osteoporotic risk. Although estrogen replacement is an established prophylaxis for OP (28), the mechanism for its protective effect has only recently begun to be understood. The identification of estrogen receptors in human and rat osteoblast-like osteosarcoma cells and in human primary osteoblast cells has provided the first indication that a direct action may be involved (29 - 31). Subsequently, it was shown that estrogen stimulates rat osteoblast cell proliferation and increases production of collagen mRNA, IGF-1 (mRNA and protein), IGF-2 and TGFβ as well as decreasing osteoblast responsiveness to PTH (32, 33). However, in normal human osteoblasts direct effects of estrogen on proliferation and differentiation were not found (34). It is possible that anti-resorptive mechanisms might predominate or that other cell targets are involved. In this regard, mRNA for the estrogen receptor has been reported in avian osteoclasts (35).

Proton Pump - The use of pH-sensitive dyes and microelectrode studies have established that bone resorption by osteoclasts is mediated by the acidic milieu in the closed space between the ruffled border of these cells and the bone surface (36). The acidic environment is responsible for activating osteoclast secreted lysosomal enzymes involved in proteolysis of the bone's organic matrix and the dissolution of its mineral. Efforts to understand the mechanism by which this acidic environment is produced have identified energy dependent proton pumps in the ruffled border (a Mg^{+2}-ATPase, a H^+, K^+-ATPase and a H^+-ATPase) as well as other ion transport mechanisms common to many cell types (a Na^+,K^+-ATPase, a Na^+, H^+ antiporter and a $Cl^-/HCO3^-$ exchanger) (37). Omeprazole, an inhibitor of gastric H^+, K^+-ATPase, was shown to inhibit bone resorption (38), and with other analogs has been recently patented for use in bone disease (39, 40). However, given the high concentration of omeprazole used, and the ineffectiveness of vanadate (a gastric H^+,K^+-ATPase inhibitor), the role of the H^+,K^+-ATPase in resorption is in question (37). The sensitivity of osteoclast acidification to N-ethyl-maleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole suggests that a vacuolar H^+-ATPase may be important (41, 42). The prevailing theory suggests that $HCO3^-$ ions produced in the osteoclast by carbonic anhydrase are passively exchanged for Cl^- at the cell surface. Chloride, in turn, serves as a counterion for a H^+-ATPase at the ruffled border (43). Recent immunohistochemical evaluation of activity has indicated that the primary osteoclast ATP-dependent proton pump (H^+-ATPase) is different from the gastric and mitochondrial proton pumps, but similar to that found in the kidney cells (44).

Calcitonin - Calcitonin is a potent inhibitor of bone resorption and is approved for clinical use in multiple skeletal disorders including OP (45). Calcitonin inhibits osteoclast motility as well as diminishing the size of the cavities they produce in bone (45). Calcitonin's inhibitory effects on rat osteoclastic bone resorption has been found to involve two separate acute actions: the abolition of cell motility (Q effect) and cellular retraction (R effect). The Q effect can be mimicked by dibutyryl cyclic AMP and cholera toxin while the R effect is mimicked by ionomycin and pertussis toxin (46). These findings suggest either mediation by at least two independent G proteins and two separate messengers, cAMP and Ca^{+2}, or a time lagged relationship of the two effects (46).

Superoxide - A defect in leukocyte superoxide generation and osteoclastic resorption in patients with osteopetrosis has been reported (47). Intracellular and extracellular superoxide generation by osteoclasts was inhibited by recombinant human calcitonin (48).

CLINICAL DEVELOPMENTS

Current and emerging options in the diagnosis and treatment of OP are summarized in Table I. Near term therapeutic advances are expected to occur as a result of refinements in administration regimens of existing agents. While this will serve to enhance acceptance of these therapies, there is a need to expand the clinical armamentarium to include novel therapies. Advances in diagnosis with the introduction of more sensitive and precise instrumentation for bone density measurement (dual X-ray absorptiometry) and the characterization of more predictive biochemical markers [collagen pyridinium cross-links (49)], are expected to facilitate the development and application of OP therapeutic regimens.

Table I. Osteoporosis Diagnosis and Treatment

Current Therapies
- Estrogen replacement
- Parenteral calcitonin
- Vitamin D and active analogs
- Calcium supplementation
- Anabolic steroids

Diagnosis
- Single and dual photon absorptiometry
- Urinary hydroxyproline and calcium; serum alkaline phosphatase and osteocalcin

Emerging Therapies
- Estrogen replacement-expanded indication
  - Perimenopause
  - Established osteoporosis
- New hormone replacement formulations
  - 17β Estradiol/Progesterone
  - Patches, implants, vaginal rings
- Intranasal calcitonin
- Bisphosphonates
- Site specific vitamin D analogs

Diagnosis
- Dual X-ray absorptiometry; urinary pyridinoline

Hormone Replacement - The value of estrogen replacement for OP prophylaxis when given at the time of menopause has been firmly established and was cited as the therapy of choice by the recent Consensus Conference on OP held in Copenhagen (28). However, there is increasing evidence that estrogen may be of therapeutic value if started later in life even in the face of pre-existing OP (50, 51). Concern that the recommended inclusion of a progestogen with estrogen replacement therapy to prevent excessive uterine stimulation might decrease estrogen's bone protective effects were allayed by studies which show that progestogens might have a positive effect on bone, independent of estrogen. (52).

Bisphosphonates (BP) - In two clinical trials on postmenopausal, osteoporotic women, intermittent cyclical etidronate therapy resulted in a significant increase in spinal bone density and a decrease in vertebral fracture rate with no major side effects and no evidence of mineralization impairment (53, 54). However, further evidence presented at an FDA advisory committee meeting indicates that fracture protective effects may be attenuated with longer administration. This and other issues, such as the effect of the long skeletal retention of BP's on bone cell activity and bone strength, remain (55). The second-generation agent pamidronate has been shown to increase bone mass in patients with steroid induced OP (56), while tiludronate was reported to prevent bone loss in a study of healthy postmenopausal women (57). Clinical results for other BP's [BM 21.0955 (58), alendronate (59), risedronate (60)] in Paget's disease and hypercalcemia of malignancy have been reported.

Calcitonin - Parenteral calcitonin is an approved therapy for OP in the U.S., Europe and Japan. Early concerns about the development of antibodies to exogenous calcitonin limiting its utility have been somewhat alleviated by studies in which a nasal delivery system produced a biological effect in patients where antibodies to exogenous calcitonin were already present (61).

Thiazide - A retrospective study, using data on women in the Framingham heart study cohort, found fewer instances of hip fracture in women on pure thiazide diuretics than in those of thiazide combination diuretics (62). A prospective study reported a 40% lower instance of fractures in women using thiazides over a ten year period than in those on other therapy (63). The Framingham data contradicts earlier work that found equivalent increases in bone mass in women using a triamterene - thiazide combination as those on thiazide alone (64). A placebo controlled trial suggested that the thiazides exert their effect primarily through improved renal calcium retention (65).

Other Therapies - Although fluoride has been demonstrated to increase bone mass (66), a recent four year prospective study in postmenopausal women argues against its utility for OP (67). In this

study cancellous bone mass was increased but there were decreases in cortical bone mass, and most importantly, an increase in fracture rate along with a significant incidence of side effects. Ipriflavone was launched in Japan in 1988 as an antiosteoporotic. Ipriflavone (600 mg/day) for one year produced increases in bone density while slowing demineralization and reducing spinal pain (68). Treatment of elderly men for six months with growth hormone produced an increase in lean body mass and lumbar vertebral bone density (69). Gallium nitrate (GN) has recently been approved in the U.S for treatment of hypercalcemia of malignancy. In a comparative study with etidronate, 86% of the patients treated with GN achieved normocalcemia during the 5 day treatment vs. only 38% with etidronate therapy (70). Studies of GN's mechanism of action have revealed that it is associated with an increase in mRNA for procollagen and osteopontin and a decrease in osteocalcin mRNA (71,72).

ADVANCES IN PRE-CLINICAL AND CLINICAL RESEARCH

Assays and Models - Table II lists the assays and models that have been most commonly used to identify antiresorptive agents. The most widely used initial screen has involved assessment of ^{45}Ca release from prelabeled neonatal bone rudiments in culture (75).

Table II. Tests For Identifying Antiresorptive Agents

Cell systems
- Rat primary osteoclast cell cultures (pits produced on bone slices) (73)
- Inhibition of osteoclast differentiation; bone marrow culture (74)

Organ cultures
- Rodent long bones and calvaria; release of ^{45}Ca (75)
- PGE_2 production by calvaria from ovariectomized rats (76)

In vivo bone resorption models
- Immobilization or disuse osteopenia (77)
- Osteopenia secondary to gonadal insufficiency (78)
  - Surgical or GnRH-induced ovariectomy in rats and monkeys (79)
  - Ovariectomized-lactating rat (80)
- Growing rat (Schenk) model (81)
- Retinoid-induced resorption model (81)
- PTH-related osteopenia in rats
  - Low calcium diet-2° hyperparathyroidism (82)
  - Exogenous PTH administration (83)
  - Hypercalcemia of malignancy; tumor models (84, 85)
- Glucocorticoid-induced osteopenia in rats (86)
- Heparin - induced osteopenia in rodents (87)
- Resorption of ectopic bone implants (88)

Recently, methodologies have been developed to assess specific osteoclast effects (bone pitting; osteoclast differentiation) (73,74). Consistent progress has been made in the refinement of existing in vivo models for bone resorption and in the identification of new models (79, 80, 88), however, they remain lengthy and labor intensive. There has been increasing evidence to support acceptance of the ovariectomized rat as a model for postmenopausal OP (78). Induction of secondary hyperparathyroidism and implantation of osteolytic cell lines have served as models for the respective clinical bone disorders (82-85).

Increased interest in the identification of bone formation inducing agents has resulted in the emergence of several novel approaches (Table III) which include methods for producing bone nodules in culture and, as a result of advances in molecular biology, techniques for tracking indices of osteoblast growth and function. Other approaches such as ectopic bone formation, which had formerly been used almost exclusively in bone factor characterization, are finding wider application (6). Due to the slowing of the bone resorptive process in aged rats, bone formation inducing effects may be more evident which has led to their suggested use as an in vivo model (100). The correlation of these models to human bone remodeling and bone disorders continues to represent a major challenge. Due to the coupled nature of the relationship between the osteoblast and osteoclast in bone homeostasis involving a complex interplay of systemic factors, a single rate limiting mechanistic target has not yet been conclusively identified. Efforts such as those aimed at regulating the proton pumping mechanism, collagenase activity, cytokine activity, etc. have yielded only modest results and suffer from a lack of bone specificity (vide infra).

Table III. Tests for Identifying Bone Formation Inducing Agents

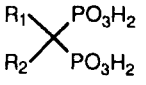
Cell systems
- Primary osteoblast cultures (89); Cell lines with osteoblast phenotype (90)
- Assess cell proliferation and functional markers (mRNA, protein) (91, 92)
  - Type I collagen, alkaline phosphatase, osteocalcin (93 - 96)
- Bone nodule formation (89)

Organ/Tissue culture
- Ectopic bone formation (bone powder implants, mandibular condyle growth) (6)
- Long bone and calvarial incorporation of ^{3}H-proline (75, 97)

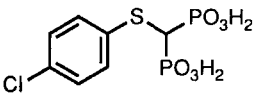
In vivo evaluation
- ^{45}Ca or ^{3}H-tetracycline incorporation in bones (98, 99)
- Histomorphometric evaluation of nondecalcified bone slices (78)
- Bone weight/volume increase in aged rodents (100)

Bisphosphonates - BP's, pyrophosphate analogues with a strong affinity for mineralized tissues, are potent inhibitors of bone resorption (101, 102). While their mode of action is still unclear, experimental evidence points to a cellular mechanism. BP's have been shown to selectively alter osteoclast metabolism through adsorption onto bone surfaces, and subsequent release and uptake by osteoclasts during bone resorption (101-104). Studies have also indicated that interference with accession of osteoclast precursors, their attachment, and their transformation into functioning osteoclasts is involved (101, 102, 105). There is also evidence that individual BP's may possess different modes of action (103).

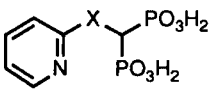
While the geminal BP group is responsible for targeting the compound to bone and is a prerequisite for activity, inhibition of resorption is dependent on the side chain (101). First generation compounds (e.g. etidronate **1**, chlodronate **2**) are simple alkyl or halogen derivatives. Lengthening the side chain (up to eight carbons) or adding a hydroxyl group to the geminal carbon increased potency. Tiludronate **3** contains a 4-chlorophenylthio-side chain (106). As inhibition of mineralization may occur with high doses of BP (e.g. etidronate **1**) (107), more potent compounds were sought to widen the therapeutic index between antiresorptive and antimineralization activity. Addition of an amine group to the side chain led to a significantly more potent second generation of BP's (e.g. pamidronate **4**). A three carbon side chain gave maximum activity (i.e. alendronate **5**). Alkylation of the primary amine is reported to boost potency as in BM 21.0955 **6** (108) and dimethyl pamidronate **7** (109).



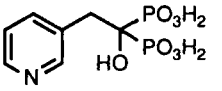
1 R_1 = OH; R_2 = CH_3
2 R_1 = R_2 = Cl
4 R_1 = OH; R_2 = $(CH_2)_2NH_2$
5 R_1 = OH; R_2 = $(CH_2)_3NH_2$
6 R_1 = OH; R_2 = $(CH_2)_2N(Me)(n\text{-Pentyl})$
7 R_1 = OH; R_2 = $(CH_2)_2NMe_2$



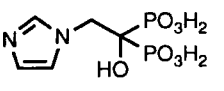
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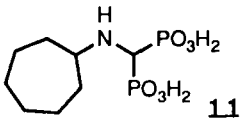
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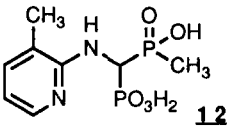
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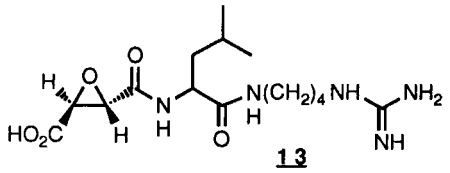


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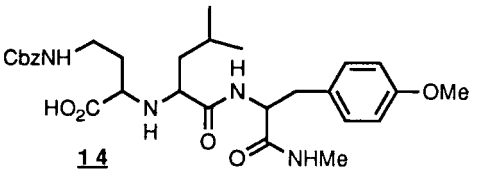
BP's with a basic, heterocyclic side chain are prototypical third generation agents (81). A series of pyridinyl BP's **8** showed a correlation of potency with linking atom X: NH>CH_2>S>O. However, a hydroxyl group at the geminal carbon led to the most potent agent **9** (risedronate, NE 58095). Highly potent imidazolyl **10** (CGP 42446) (110), and cycloheptylamine **11** (YM 175) (111) analogues have been reported. As increased affinity for hydroxyapatite correlates with an increased inhibition of mineralization, the correction of this potential defect by designing analogs

with decreased hydroxyapatite binding has been explored (112). Phosphonoalkyl phosphinates (e.g. **12**) are reported to possess diminished hydroxyapatite-crystal growth inhibition, while maintaining in vivo bone resorption inhibitory activity. However, their potency is greatly diminished in comparison to the corresponding BP's (112). The antiresorptive activity of BP's have been compared in the growing rat model (81). First generation agents have activity in the dose range 0.1 to 10 mg P/Kg, s.c., second generation agents in the range 0.01 to 1 mg P/Kg, s.c. and third generation agents have shown activity as low as 0.001 mg P/Kg, s.c..

Enzyme Inhibitors - While the activity of omeprazole in remodeling has focused attention on the ATPase enzymatic targets for new drugs other enzymes have similar potential. Recent work suggests that inhibition of the Na^+/H^+ antiporter (using amelioride) in osteoclasts is important in slowing resorption (37). Collagenase and other cysteine proteinases are also being evaluated as mediators of resorption. Collagenase is secreted by osteoblasts as procollagen in response to resorptive agents such as PTH, 1, 25 $(OH)_2$ D_3 and vitamin A (113). Collagenase activity is regulated by an endogenous irreversible inhibitor, tissue inhibitor of metalloproteinases (TIMP). This substance, first isolated from rabbit bone culture, has a molecular weight of 28Kd and inhibits several metalloproteinases besides collagenase (114). The presence of collagenase and cysteine protease in bone tissue and the isolation of TIMP suggested the potential use of inhibitors of cysteine proteases [e.g. Z-PheAlaCHN_2 and E64 (**13**)] (115-117) and collagenase (e.g. CI-1, **14**) (118) in the treatment of OP. The cysteine proteinase inhibitors significantly reduced pitting by chicken osteoclasts in dentin (119). E64 was orally active in a rat secondary hyperparathyroidism model at 40 mg/kg (117).



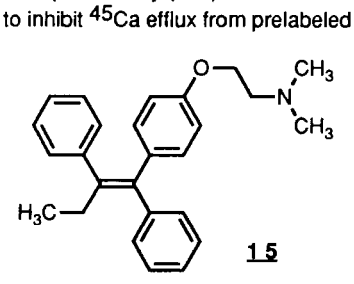
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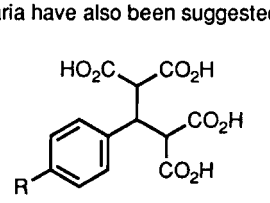
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Prostaglandins - Prostaglandins, particularly those of the E series, have been shown to be involved with increases in both bone resorption and formation (120). PGE_2 has been shown to stimulate longitudinal bone growth in intact or ovariectomized female rats, an effect not seen in male rats (121). A correlative study showed PGE_2 activates intracortical bone remodeling and cortical bone formation. PGE_2 resulted in more intracortical bone remodeling, cancellous bone formation and periosteal bone growth in ovariectomized rats than in intact PGE_2 treated rats (122).

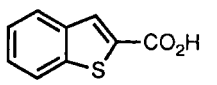
Miscellaneous - Novel vitamin D analogs continue to attract attention for use in OP. For example, a combination of cholecalciferol derivatives has recently been patented in Europe (123). The antiestrogen, tamoxifen **15**, was reported to prevent trabecular bone loss due to ovariectomy in rats at doses that did not stimulate uterine weight gain (124). Recently tamoxifen been shown to increase bone mineral density in women receiving it as treatment for breast cancer (125). The γ-carboxy glutamic acid residues of osteocalcin inspired the design of polymalonic acids (e.g. **16**) as bone affinity agents (126). These compounds may be used as drug delivery agents by linkage of drugs which modulate bone resorption or formation to the aromatic R group. Tetracycline or BP's covalently linked to carbonic anhydrase inhibitors have been reported for the treatment of bone disorders (127). Benzothiophene carboxylic acid **17** is in development for hypercalcemia of malignancy (128). A series of benzothiadiazines (e.g. **18**) were found active in a PTH stimulated resorption assay (129). Two related series of quinolines (e.g. **19** and **20**) which have been shown to inhibit ^{45}Ca efflux from prelabeled calvaria have also been suggested for use in OP (130, 131).



15

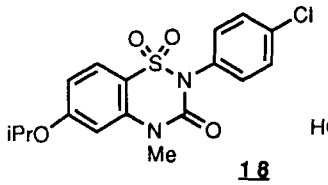


16 R = NH_2, CH_2NH_2, CO_2H

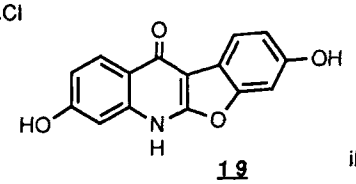


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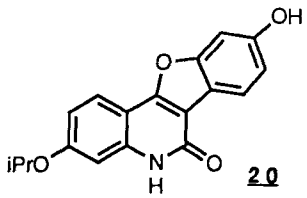
Two stilbene derivatives (21) which are known inhibitors of cellular anion exchange were reported to be potent inhibitors of resorption in fetal rat bones in culture (132). Tris perchlorate (10mM) was shown to reduce in vitro bone resorption. This was shown to be unrelated to enzymatic inhibition, but it was observed that tris perchlorate inhibits osteoclast spreading (133). Zeolite was reported to increase ^{3}H-thymidine uptake, alkaline phosphatase activity and release of osteocalcin induced by 1,25(OH)$_2$ D$_3$ in human osteoblasts (134). The dihydropyridine calcium agonist, BAY K 8644 (22), was found to stimulate osteocalcin production by osteoblast-like cells in culture but in bone organ culture its net effect was to stimulate bone resorption (135).



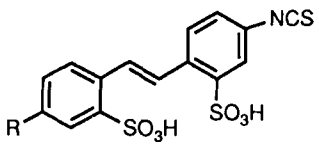
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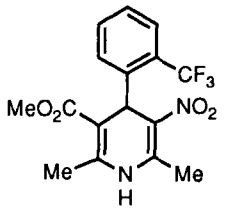
19



20



21 R = NCS or CH_2CONH_2



22

Conclusion - The elaboration of various paracrine and autocrine components of the bone metabolic regulatory complex and further understanding of the molecular and cellular events surrounding bone cell proliferation, differentiation and function represent major research achievements of the last few years. The availability of these new regulatory factors (through recombinant DNA technology) should enable significant advances in our understanding of bone biology and help to define targets for future therapeutic approaches.

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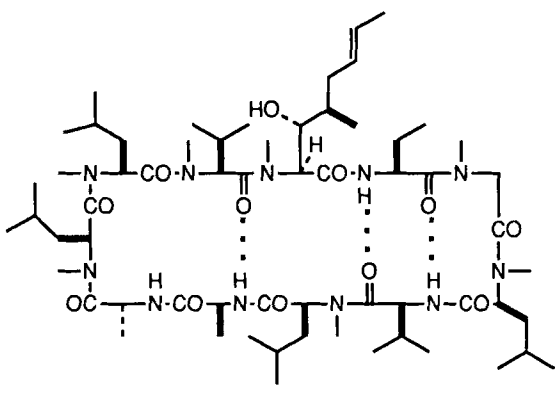
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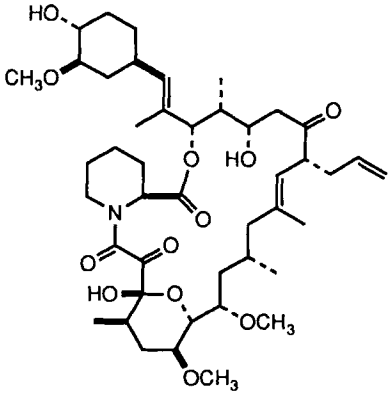
Chapter 22. Mechanism-Based Immunosuppressants

Jay R. Luly
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Introduction - Since cyclosporin A (CsA, 1) ushered in a new era of immunosuppression in the 1980's, the field has witnessed an intense exploration for mechanistic understanding as well as a persistent search for new and superior agents. In this rapidly evolving area, other macrocyclic immunosuppressants have emerged, including the potent macrolactam FK-506 (2), as well as several other classes of considerable interest, both structurally and mechanistically. This chapter will offer a brief summary of recent literature in this dynamic area of research.



CsA, 1



FK-506, 2

MACROCYCLIC IMMUNOSUPPRESSANTS

FK-506 - The macrolactam antifungal FK-506 was discovered in 1984 by workers at Fujisawa in a *Streptomyces tsukubaensis* culture while screening for compounds capable of interfering with the production of IL-2 (1-3). Numerous synthetic studies have culminated in 3 recent total syntheses (4-7). Degradative and semi-synthetic modifications have also been described (8-10). Reviews covering a significant portion of the early biology related to FK-506 have appeared (11-16).

Studies have demonstrated that FK-506 is superior to CsA in several allograft models including renal and liver transplants in dogs (17), as well as small intestine (18) and heterotopic heart transplants in rats (19, 20). In cynomolgous monkeys, prolonged liver (21), kidney (17), pancreatic (22), and cardiac allograft survivals (23) have been reported. FK-506 was found to be more potent than CsA in murine models of accelerated ear-heart graft rejection and antigen-stimulated lymphoproliferation (24, 25). Efficacy in insulitis and diabetes in non-obese diabetic mice (26, 27), spontaneous diabetes in BB rats (28), collagen-induced arthritis in rats (29), as well as other models of autoimmune disease (30) have been reported. Early results in xenograft recipients however, are not encouraging (31, 32).

Although FK-506 has been shown to be more potent than CsA in a variety of *in vitro* and *in vivo* models, a number of species variations have been documented (33). For example, the baboon is 10-fold less sensitive than the rat or dog (34). The potency of inhibition of the mixed lymphocyte reaction (MLR) was 3.8, 0.33, 0.29, 0.59 ng/mL in the baboon, rat, human, and dog, respectively. Differential species toxicity has also been observed (*vide infra*).

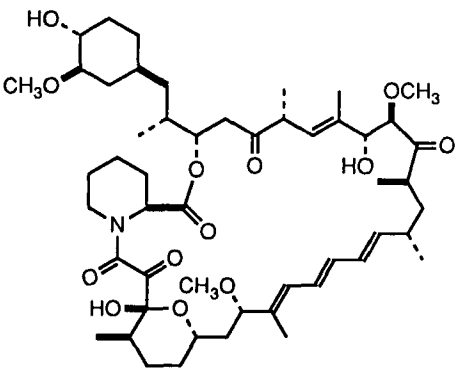
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Since the first reports of early clinical success in liver transplant patients (35-37) and recent reports of success in kidney (38), and thoracic organ transplantation (39), the compound has received a considerable amount of attention. Recently eighteen clinical papers were contiguously published (40).

Despite these encouraging early results and the fact that FK-506 is reasonably well tolerated, adverse effects (33, 41) have been observed in a number of species, including hyperglycemia in rats (42) and baboons (43). Lethal emaciation in the latter species has been noted after i.m. administration (43); however, other investigators reported no major toxicity after oral administration of 2 to 18 mg/kg/d (44). In the dog, anorexia and vascular changes in the heart and gastrointestinal tract (45-47) have been reported. The degree and frequency of these toxic effects are species dependent, being minimal in rodents and severe in dogs (33). To date, the drug seems to be fairly well tolerated in humans (48, 49). Adverse side effects, which are worse upon i.v. administration, resemble those of CsA and include nausea, vomiting and headaches (48). Tremors, an increased sensitivity to light, burning or tingling sensations in the hand and foot, insomnia, and mood change were among other variably reported side effects (38). Although the clinicians have also reported limited nephrotoxicity and new-onset diabetes, there was a notable absence of hypertensio.., hirsutism, gingival hyperplasia, and gynecomastia, and their preliminary observations suggest a more advantageous therapeutic index than CsA (39).

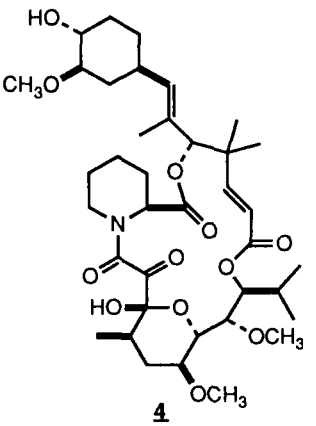
Rapamycin - Another antifungal macrolactam, **3**, was isolated in 1975 by workers at Ayerst (50, 51). Although the immunosuppressive properties of **3** were first recognized in 1977 (52), it was reports of the clinical success of FK-506 that prompted reinvestigation of **3** for this utility (53-55). While **3** is structurally similar to FK-506, it is reported to have a number of distinguishing mechanistic properties. Rapamycin potently inhibits many types of stimulated lymphocyte proliferation *in vitro* (100-1000 times more potent than CsA) (56-58) as well as alloantigen-stimulated lymphoproliferation *in vivo* (59). It is more potent than CsA in several models of autoimmune disease including experimental allergic encephalomyelitis (EAE) (52), systemic lupus erythematosus (SLE) (60), methylated bovine serum albumin (MBSA)-induced delayed-type hypersensitivity (DTH) (61), collagen-induced arthritis (62), as well as developing and established rat adjuvant arthritis (61). In the murine graft vs host (GvH) reaction, **3** was more potent than either FK-506 or CsA, while in the host vs graft (HvG) reaction it was more potent than CsA, but less potent than FK-506 (59). It has been shown to be more efficacious than FK-506 or CsA in models of mouse ear-heart and rat whole heart allografts, prolonging survival significantly with a single 12 mg/kg i.p. dose (63, 64). In a rat heart allograft model, indefinite graft survival (63) and an induction of antigen-specific unresponsiveness (59) were produced by **3**. Rapamycin also produces a more potent, dose-related prolongation of mouse skin grafts compared to CsA; either agent provided a mean survival time of 10-11 days at doses of 0.25 and 32 mg/kg i.p. or 2.5 and 100 mg/kg p.o., respectively (65). Little has been reported on the toxicity of **3**; acute necrotizing fibrinoid vasculitis of the arterioles and small arteries as well as gastrointestinal tract ulcers are the major side effects observed in dogs after oral dosing with **3** (53).



Rapamycin, **3**

Mechanistic Aspects of CsA, FK-506, and Rapamycin - Although CsA has been widely and successfully used in patients undergoing organ transplantation since the early 1980's, understanding of its mechanism of action and toxicity is still evolving. Despite their dramatically different structures, CsA and FK-506 share many common immunosuppressive properties (13, 16). The early phase of $CD4^+$ T-cell activation (66) and lymphokine production (67-70) seem to be important targets for the inhibitory effects of CsA and FK-506 (71). Further, the effects on transcription exerted by CsA (72-74) and FK-506 (73, 74) are mediated in part by blockade of nuclear transcription factors, such as nuclear factor for activated T-cells (NF-AT), that bind to sites on the IL-2 enhancer (75,76). While CsA and FK-506 (but not rapamycin) inhibit the transcription of early phase T-cell activation genes (IL-2, IL-3, IL-4, IFN-γ, TNF-α, GM-CSF) (67, 69), rapamycin (but not CsA or FK-506) is reported to exert its effect by rendering T-cells unresponsive to the proliferative effects of IL-2 (57). There is, however, recent evidence that rapamycin can inhibit

lymphokine production (IL-2, IL-4, IFN-γ and, to a lesser extent, IL-6) in human PBMC stimulated with Con A (77) at a step other than transcription (69). Thus, rapamycin appears to have inhibitory effects on both lymphokine production, as well as lymphokine-stimulated T-cell activation.

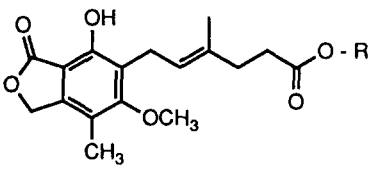


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The fact that CsA and FK-506 avidly bind to different cytosolic proteins, cyclophilin (78) and FK binding protein (FKBP) (79-81), respectively— both of which possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity specifically inhibitable by their respective ligand (80-83), raises interesting questions about the possible similarities and differences in their mode of action and toxicity (84). Because the PPIase activity has been demonstrated to play a key role in the catalysis of protein folding (85), mechanistic studies have been undertaken to understand this process in greater detail. Early investigations proposed that the isomerase catalyzed *cis-trans* amide bond interconversion via nucleophilic addition of a cysteine sulfhydryl group to the amide (83). Systematic site-directed mutagenesis of cysteine residues in cyclophilin, however, resulted in preserved enzymatic activity (86), suggesting that this is not the case. One recent study, which used succinyl-Ala-Xaa-Pro-Phe-*p*-nitroanilide (Xaa= Ala, Gly) as a cyclophilin substrate, indicated that catalysis was instead mediated by distortion of the Xaa-Pro amide bond (87)— a region that the tri-carbonyl portion of FK-506 has been proposed to mimic (88, 89). An NMR study examining the FKBP-catalyzed *cis-trans* interconversion of this substrate (Xaa= Ala) has recently been detailed (90). Variations in Xaa in this substrate have revealed a dramatic difference in substrate specificity for cyclophilin and FKBP (91). That FKBP PPIase inhibition is not sufficient for immunosuppressant activity was shown by study of an analogue of FK-506 and rapamycin, 506BD (**4**). This analogue, which lacks immunosuppressive activity, not only binds avidly to FKBP and inhibits its enzymatic activity, but it also blocks the immunosuppressive activity of FK-506 and rapamycin (92). A recent report provides evidence for a common cytosolic binding protein for CsA and FK-506 (93).

Mixtures of various immunosuppressive drugs have long been used to study combination effects on a number of different immunosuppressive pathways. For example, combinations of rapamycin and CsA synergistically prolong skin (65) or heart allografts (59), and synergistically inhibit stimulated proliferation of T-cells (94-96). This is in contrast to the behavior of rapamycin and FK-506, which have been reported to be antagonists of each other's effects *in vitro* (94, 97). Interestingly, while these *in vitro* studies under defined conditions suggest reciprocal antagonism of rapamycin and FK-506, *in vivo* they were found to behave synergistically in prolonging mouse heart allograft survival (59).

IMMUNOSUPPRESSANTS BLOCKING NUCLEOTIDE SYNTHESIS



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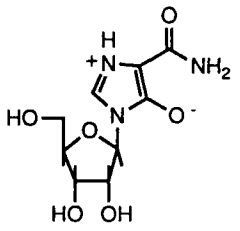
6 R =

Mycophenolic acid and RS-61443 - Mycophenolic acid (**5**), a phenolic fungal metabolite with known anti-cancer (98, 99), antiviral (98, 100), and immunosuppressive effects (101, 102), is an inhibitor of two enzymes, inosine monophosphate dehydrogenase (IMP-DH) and guanylate (GMP) synthetase (103-106), involved in the biosynthesis of guanosine by the *de novo* pathway. T-cells and B-cells, which lack the salvage pathway of purine synthesis found in other cells, are uniquely dependent on the *de novo* pathway (107, 108), and are therefore selectively targeted by **5** and its morpholinoethyl ester, RS-61442 (**6**). Mycophenolic acid inhibits mitogen induced T- and B-lymphocyte proliferation in the range of its IC_{50} for IMP-DH inhibition (20-60 nM). Data in these assays for side chain analogues have been recently reported (109).

Analogue **6** has been successfully used to prolong allograft survival: islet in mice (110), heart in mice and rats (111, 112), kidney in dogs (113, 114), and heart in monkeys (115). Compound **6** was also found to reverse ongoing rat heart allograft rejection (112), to induce strain-specific graft tolerance (110, 115), and to be at least additive when combined with either CsA or FK-506 in rat heart allograft prolongation (112, 116, 117). The parent drug **5** entered clinical trials for psoriasis,

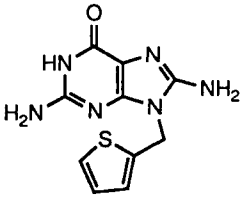
but was discontinued in 1977 due to the immunosuppressant potential of the drug as well as acute gastrointestinal side effects; however, the overall side effect profile over the course of 13 years of human dosing was not severe (118). Recently, **6** entered the clinic in renal transplantation (113).

Mizoribine (Bredinin) - Mizoribine (**7**) is an imidazole nucleoside first isolated from *Eupenicillium brefeldianum* in 1971 as a metabolic inhibitor of purine synthesis (119) that was first used clinically in Japan in the early 1980's (120, 121). Although it gives only marginal prolongation of rat skin allografts when compared to CsA and FK-506 (20), it has been successfully used synergistically in combination with CsA in models of canine kidney, pancreas, and heart transplantation (122-124), and has successfully replaced azathioprine in a triple therapy along with CsA and steroids in a clinical trial of renal transplantation resulting in reduced incidence of bone marrow suppression and systemic infections (125). Mizoribine is thought to be converted by adenosine kinase to its 5'-monophosphate derivative (126) which then exerts its inhibitory effects in a manner similar to **5** and **6** by inhibition of IMP-DH and GMP-synthetase (126-128). Erosive changes in the bowel mucosa were the major side effects in dogs (129, 130). A report of the synthesis of 5'-alkylphosphates of **7** has recently appeared (131).



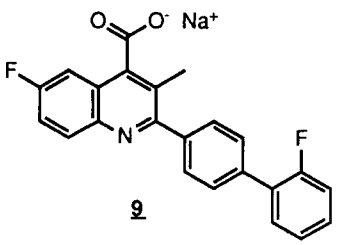
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Purine Nucleoside Phosphorylase (PNP) Inhibitors - The congenital deficiency of PNP results in both a selective and dramatic T-cell depletion that is mediated by its substrate, 2'-deoxyguanosine. The prospect for targeting this portion of the lymphoid compartment selectively, thereby affecting cell-mediated but not humoral immunity, has led to the search for inhibitors of this enzyme as novel immunosuppressants. A review on the related biochemistry, enzymology, and SAR has recently appeared (132). An 8-amino-9-heteroarylguanine, PD-119,229 (**8**), potently inhibits (Ki= 67 nM) human erythrocyte PNP (133).



8

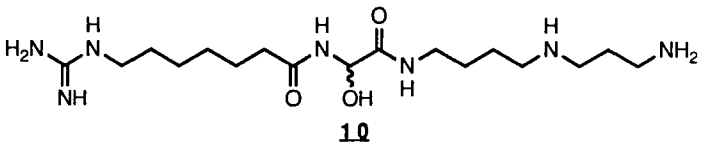
Dihydroorotate Dehydrogenase (DHO-DH) Inhibitors - Brequinar sodium (DuP-785, NSC-368390, **9**) inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, the fourth enzyme in the *de novo* pyrimidine biosynthetic pathway leading to formation of UMP. Structure activity studies performed on this series revealed that lipophilicity at C2, a carboxylic acid at C4, and the fused benzo portion of the quinoline were important for the maintenance of good enzyme inhibition (134). Although side effects in humans, including thrombocytopenia, mucositis, skin rash, myelosuppression, and nausea have been observed in Phase I cancer trials with **9** (135-138), promising results in the suppression of adjuvant-induced arthritis in rats (139) and chemically-induced hyperplasia in mouse skin (140) after oral dosing were recently reported.



9

MISCELLANEOUS IMMUNOSUPPRESSANTS

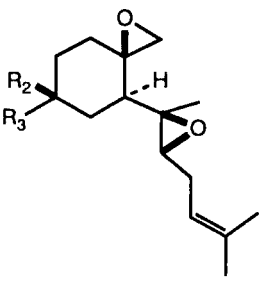
15-Deoxyspergualin - A derivative of the antitumor antibiotic spergualin (141), 15-deoxyspergualin (**10**) was first synthesized in 1982 (142). Numerous reports have appeared of immunosuppressive activities of **10** in transplantation of rat skin (143, 144), liver (145), heart (146, 147), and islet (148) grafts. Efficacy of **10** in models of autoimmune disease has also been described: SLE in mice (149-151), EAE in guinea pigs (152) and rats (153), and HvG (154) and GvH (155, 156) disease in mice. Toxicity has been observed in dogs undergoing renal transplantation and, to a lesser extent, in rats undergoing heart transplantation (157). Reversible weight loss in mice has also been reported (154). Deoxyspergualin has been shown to prolong rat heart graft survival for more than 100 days at doses comparable to CsA, 2.5 or 5 mg/kg (147), and to be six to seven times more potent than CsA in prolonging mouse heart graft survival (154). It is less potent than CsA however, in inhibiting *in vivo* lymphoproliferation (154).



10

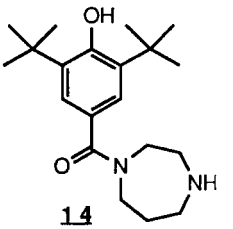
Analogues of spergualin modified at the α-hydroxy glycine residue and guanidine tether have been synthesized and tested for antitumor and immunosuppressive activity (158). Differential effects on their behavior in L1210 (IMC) mouse leukemia, DTH, and plaque forming cell assays relative to **10** were seen with a variety of analogues, most of which contained Gly or Ser at the central residue. Although little is known about the specific mechanism of action of **10**, it appears to interfere with antigen-presenting cells during the induction phase of allograft responses and to reduce the expression of graft MHC class I antigens (159). Study of a methylated analogue of **10** suggests that IL-2 receptor expression and cytotoxic T-cell generation, but not PHA- or OKT3- induced blastogenesis are also sites of inhibition (160).

Fumagillin Analogues - Fumagillin (**11**) is an antibiotic isolated from *Aspergillus fumigatus* cultures (161, 162). The structure determination (163, 164) and total synthesis in racemic form have been reported (165), as well as a variety of biological effects, including amebicidal activity and antiprotozoal activity (162). More recently, its saponification product, fumagillol **12**, and a novel analogue FR65814 (**13**) obtained from *Penicillium jensenii* F-2883, have been shown to possess immunosuppressive activity (166). Although the mechanism of action has not yet been elucidated, compounds **12** and **13** exert their effects in a manner different from FK-506 in that they weakly inhibit the growth of IL-2 independent EL4 lymphoma and do not inhibit IL-2 production (166). Interestingly, **11** was reported to be an inhibitor of angiogenesis (167), and patent applications on related analogues of **12** have published for this use (168, 169).



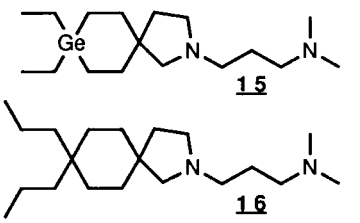
	R1	R2	R3	E
11	CH_3	H	O-CO(CH=CH)$_4$CO$_2$H	
12	CH_3	H	OH	
13	H	OH	H	

Homopiperazine Derivatives - A85 4777 (**14**) was recently reported to be effective in inhibiting models of adjuvant arthritis and the reverse passive Arthus reaction in rats as well as acute and chronic GvH disease in mice (170). It was also shown to be effective in prolonging mouse skin graft survival (171) using 5-20 mg/kg (i.p. or p.o.) and heterotopic heart allograft survival (172) using 15 mg/kg (i.p. or p.o.). However, its mechanism of action is unknown and clearly non-classical in that it has no immunosuppressive effect on the development of antibody-producing cells to the T-cell antigen SRBC or on the mitogen stimulated blastogenesis of T-cells (170).



14

Suppressor Cell Inducing Agents - Spirogermanium (**15**) is an antitumor agent (173) that has undergone extensive Phase I (174-176) and Phase II (177-180) studies for a variety of malignancies. The principal clinical toxic effects observed were neurologic (174-176), manifested as lethargy, dizziness, tremors, confusion, and ataxia. Myelosuppression, nausea, or gastrointestinal side effects were not observed (177). More recently, **15** has been shown to induce suppressor cell populations similar to the natural suppressor cell induced by total lymphoid irradiation, and to be efficacious in adjuvant arthritis, EAE, and DTH models (181-186). Extensive structure activity relationships of a series of **15** analogues have appeared (187), and a non-germanium-containing analogue, SK&F 105685 (**16**), was found to be more efficacious than **15** in models of AA, EAE, and suppressor cell activity at 30 mg/kg p.o. (187, 188). Further, a positive correlation between the activity in the AA model and the ability to induce suppressor cells was observed among several analogues (187, 188). It has been recently reported that (**16**) is effective at 20 mg/kg p.o. in prolonging rat heart allografts, and that its effects are synergistic in combination with CsA (189).



15

16

Future Directions - Significant progress has been made in the identification of new and mechanistically distinct immunosuppressants. While it is known that these compounds likely exert their effects via a variety of different paths, the total elucidation and refinement of their mechanism(s) of action is ongoing. Toxicity of varying degree is a common property of most immuno-suppressants. Thus, the utility of traditional, clinically-used immunosuppressants is largely

limited to transplantation, and the array of autoimmune disorders awaits a safer drug. In this exciting period of immunosuppressant research, potent, and perhaps less toxic, compounds are emerging which may be useful as single agents, or alternatively as components in synergistic combinations.

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SECTION V. TOPICS IN BIOLOGY

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Chapter 23. Cytokine Receptors

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Introduction - Cytokines are the proteins which are responsible for the regulation of the growth, differentiation and activation of the hematopoietic cells responsible for the maintenance of the immune system. A description of the biological activities associated with each of these molecules is beyond the scope of this article, but can be found in recent reviews (1,2). Our understanding of the means by which cytokines stimulate target cells has been greatly aided by the elucidation of the cell surface receptors to which the cytokines bind. Cytokine binding studies have been recently described in some detail (3,4) and have served to define the variety of functions which many of these regulatory factors may possess. Cytokine receptors are not exclusive to cells of the immune system, suggesting that cytokines may play roles in other organ systems quite distinct from those that we have come to identify for them within the immune system. With the cloning of the genes which encode the cytokine receptors, has come the realization that the receptor proteins may be grouped into discrete families based on regions of sequence similarity shared between different receptor genes. Perhaps most intriguing of all has been the discovery that many of the cytokine receptors exist as different isoforms, some of which are soluble. The existence of soluble cytokine receptors provides a natural means by which the stimulatory effects of cytokines on the immune system may be regulated, and has provided a rationale for the examination of their potential pharmacologic activity.

CYTOKINE RECEPTOR FAMILIES

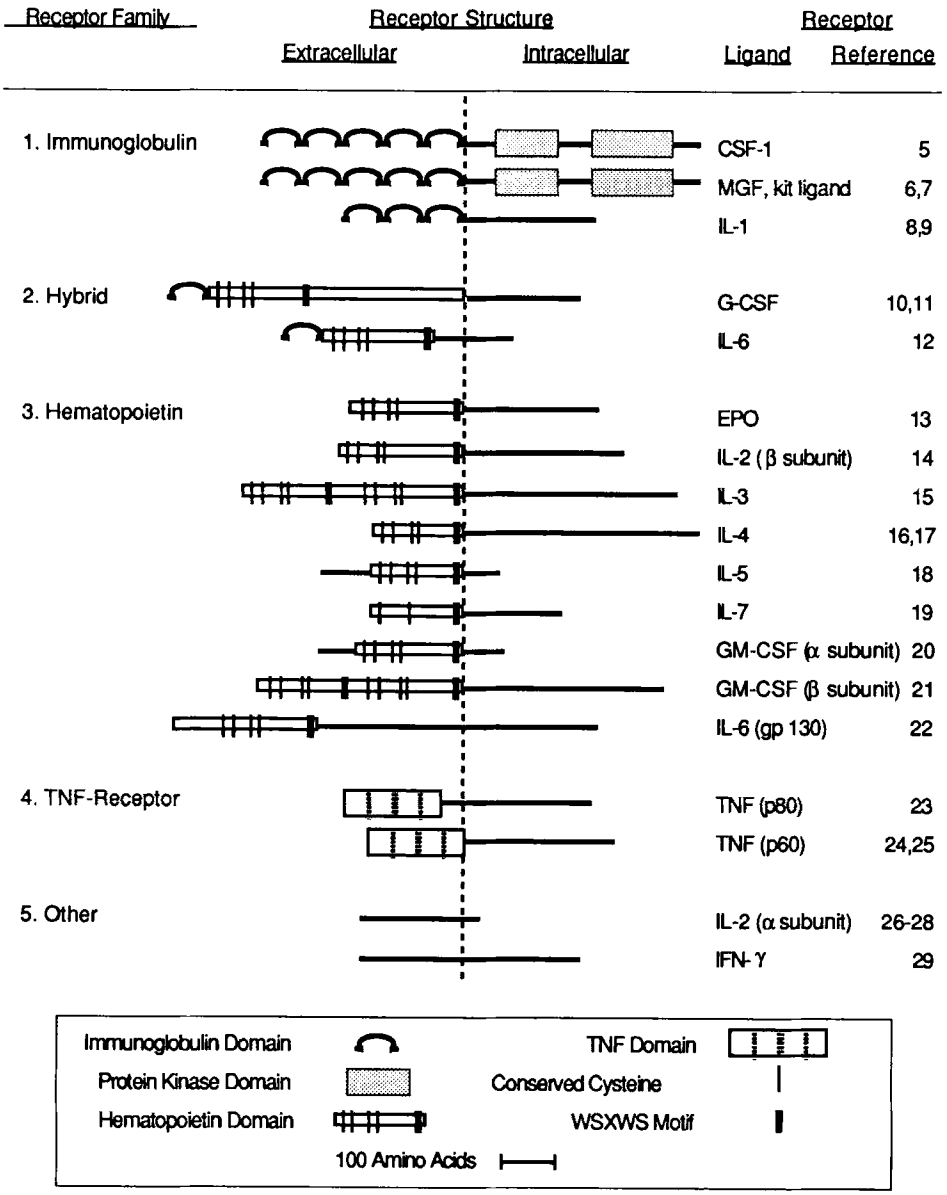
The genes encoding the cytokine receptors have been cloned by a variety of techniques which have ranged from the purification of the native receptor protein to the direct expression of cDNA libraries in cells to which tagged cytokines or antibodies could then bind. These gene cloning activities have occurred at a rapid pace and similarities in the predicted amino acid sequence of the receptors have allowed them to be grouped into families as shown in Table 1. Thus, the CSF-1 receptor; kit ligand or mast cell growth factor receptor, c-kit; and interleukin 1 (IL-1) receptor have extracellular domains composed of 3 to 5 immunoglobulin-like domains. Immunoglobulin domains are characterized by an antiparallel β sheet structure, two layers of which are anchored by an intra-chain disulphide bond buried in the core. Confirmation that the three Ig-like domains in the IL-1 receptor exist as discreet units, has come from studies in which DNA encoding each of the domains has been deleted from the complementary DNA encoding the IL-1 receptor. The resulting mutants were expressed in cells and while protein expression was confirmed using antibodies directed to the IL-1 receptor, it was clearly shown that IL-1 bound to the receptor only when all three Ig-like domains were expressed in the same molecule (30). Cell surface proteins having Ig-like domains include the B cell antigen receptor (surface immunoglobulin), the T cell antigen receptor, both class I and class II MHC molecules, CD4, CD8 and cell adhesion molecules such as NCAM (31). Each member of this rapidly growing family is involved in protein-protein, protein-antigen or cell-cell interactions, emphasizing the critical role that this type of domain plays in these specific recognition events. The CSF-1 receptor and c-kit are distinguished from the IL-1 receptor by having intracellular domains reminiscent of the tyrosine kinase structures.

Two receptors which contain a single immunoglobulin like domain are the receptors to which the cytokines, granulocyte colony stimulating factor (G-CSF) and IL-6 bind. They have been segregated from the other members of the immunoglobulin supergene family in Table 1 because

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Table 1
Cytokine Receptor Families



they also have domains sharing significant similarity to the primary extracellular domain of the hematopoietin receptor family. This domain is characterized by having a pentapeptide sequence Trp-Ser-X-Trp-Ser at the 3' end of the domain unit and conserved cysteine residues localized at the 5' end of the protein. New members of this family have been described at a phenomenal rate and the family now consists of the receptors to which erythropoietin (EPO), IL-2 (the β subunit), IL-3, IL-4, IL-5, IL-7, GM-CSF and G-CSF bind. The family also contains the genes which encode subunits that interact with cytokine receptors. The second subunit of the GM-CSF receptor and gp 130, the second subunit of the IL-6 receptor, do not bind ligand directly, but associate with the GM-CSF receptor and IL-6 receptor to confer high affinity binding and signal transduction respectively, on those receptor systems. The family also contains the receptors to which prolactin (32, 33) and human growth hormone bind (34).

A recent review discusses the structural features of the hematopoietin familiy of receptors in extensive detail (35). Studies on the structure of the growth hormone receptor (36) have resulted in the determination that the first and second cysteine residues are linked and the third and fourth cysteine residues are linked in disulfide bonds. If one uses the structure of this receptor as a model for this family then one would predict that a similar two looped motif exists in other members of the family. Analysis of the sequence of the hematopoietin domain has also revealed that the carboxy terminal region of the hematopoietin domain is homologous to the fibronectin type III domain (37). This domain was originally described as 90 residues in length and is repeated 15 times in fibronectin (38). Its location in the IL-4 receptor coincides with the site at which alternative RNA splicing has been shown to occur (16). It is interesting to note that the Trp-Ser-X-Trp-Ser sequence occurs in the hematopoietin domain in the area which is analogous to the location of the Arg-Gly-Asp sequence in the fibronectin domain. The Arg-Gly-Asp sequence is well recognized as the region which mediates the association of fibronectin with the cell surface fibronectin receptor (39). It is thus intriguing to speculate that the Trp-Ser-X-Trp-Ser region of the hematopoietin family may mediate the association of these receptors with other receptor subunits, as in the case of the GM-CSF, IL-6 and IL-2 receptors or with other proteins and constituents on the cell surface. In particular, fibronectin interacts with heparin, which may provide an explanation for the reports that colony stimulating factor activity has been found associated with the extracellular matrix (40, 41).

A new family of cytokine receptor genes was identified with the cloning of the p80 and p60 subunits of the tumor necrosis factor (TNF) receptor. The subunits are structurally related at the primary amino acid level. The extracellular region shows the most significant degree of similarity primarily due to the presence in this region of 22 conserved cysteine residues. This region can be divided into four segments of approximately 40 residues in length which display a similar sequence motif, suggesting that these genes may have evolved by gene duplication of a smaller ancestral gene. The sequence similarity and internal structural homology found in the TNF receptor subunits is also found in the gene which encodes the receptor to which nerve growth factor binds (42).

The alpha subunit of the IL-2 receptor and the receptor to which IFNγ bind are not related to each other or to members of the other families depicted in Table 1.

The identification of Ig-like, hematopoietin and fibronectin-like domains in the receptor molecules (all three are present in the hybrid receptor family depicted in Table 1) suggest that, like other receptor families, the extracellular portion of these receptors was assembled from genetic modules (37), the mixing of which provides functional diversity to the evolution of these families.

SIGNAL TRANSDUCTION

Although the identification of the receptors to which the cytokines bind has occured at a rapid pace, our understanding of the signals which are engendered upon occupation of a receptor with its cytokine ligand remains several steps behind. Examination of the sequences of the cytoplasmic domains of the receptors depicted in Table 1 reveals that only two (CSF-1 receptor and cKit) of the eighteen receptors described, have regions remininscent of domains known to be involved in signal transduction via tyrosine phosphorylation. The cytoplasmic domains in many of the other receptors depicted in that table are large enough to have enzyme functions, but few clues to such an activity can be generated from the analysis of their protein sequence.

It is clear however, that these regions are likely to be required for signal transduction. Transfection experiments with the IL-1 receptor, for example, have shown that IL-1 responsiveness can be conferred to cells by introduction of a complete IL-1 receptor into the cells. If the cytoplasmic domain has been deleted from the receptor gene prior to the transfection of the gene into cells, then IL-1 binding on cells can be demonstrated, but IL-1 responsiveness is absent (43). Beyond

knowing that this region is necessary for signal transduction; little else in this system is known with certainty, but many signaling systems have been examined in some detail (44). These studies suggest that IL-1 can activate protein kinase A, protein kinase C, and G-proteins (both pertussis sensitive and insensitive); stimulate Na^+/H^+ exchange and activate a serine/theronine kinase distinct from kinase A or C. The role that these systems play in IL-1 signal transduction has been recently debated in some detail (45, 46). One area which is not as controversial as the signal mechanism is the recognition that IL-1, like TNF, affects the expression of specific genes in target cells by activating transcription factors that bind to AP-1 or $_{k}$B sites in the 5' regulatory regions of the affected genes.

As mentioned in the previous section, several of the cytokine receptors are composed of more than one subunit. The GM-CSF receptor, for example, is composed of α and β subunits, the combination of which results in the capacity to bind GM-CSF with high affinity. This association may not, however, be required for signal transduction since expression of the low affinity α subunit alone appears to be sufficient to engender responsiveness to human GM-CSF in murine cells (47). In contrast, the IL-6 receptor depicted in Table 1 will only transduce a signal when the second subunit of this receptor, gp 130, is present in the cell. The two subunits are thought to associate via regions on the extracellular domains since a soluble form of the IL-6 receptor, in the presence of IL-6, was shown to bind and transduce a signal through the gp 130 subunit (22). The receptor for IL-3 is thought to associate under some conditions with the GM-CSF receptor as evidenced by the capacity of GM-CSF and IL-3 to compete for binding sites on cells to which both GM-CSF and IL-3 bind (reviewed in 4). The IL-3 receptor that has been cloned (15), binds with low affinity suggesting that a second subunit might be required to confer high affinity binding. The IL-3 receptor may associate with the same second subunit to which the low affinity GM-CSF receptor associates and thus explain the ligand competition results. Recent studies on signal transduction with IL-3 and GM-CSF support this symmetry and suggest that both GM-CSF and IL-3 stimulate the same pattern of tyrosine phosphorylated proteins in target cells (48). Both IL-3 and GM-CSF have also been reported to induce the phosphorylation of serine residues found in the Raf-1 proto-oncogene (49).

The information about how cytokine receptors deliver signals is far from complete but the collection of cytokine receptor genes provides us with the tools that are needed to build an understanding of this process.

SOLUBLE CYTOKINE RECEPTORS

The characterization of the genes which encode the cytokine receptors has led to the realization that many of these genes can yield several different isoforms of the receptor proteins by alternative exon splicing mechanisms. The most intriguing isoforms are those in which the transmembrane region is absent, resulting in a receptor molecule which can be secreted from cells. The existence of such isoforms was discovered by the isolation of several different cDNAs which encode the receptors. Analysis of the nucleotide sequence of these cDNAs indicated that the sequences of the cDNA's diverged at various locations creating cDNAs of different length and different 3' terminal sequences. This observation was first made with the IL-4 receptor (16). The cDNAs encoding this receptor were cloned from a cDNA library prepared from mRNA isolated from a murine cytotoxic T cell line. One class of cDNA encoded a polypeptide of 810 residues which contained a putative signal sequence of 25 hydrophobic residues at the amino terminus. This region was followed by a sequence of 208 residues, thought to represent the extracellular region, in which five potential asparagine linked sugar substrates and five cysteine residues were located. A stretch of 24 hydrophobic amino acids follows this region and is of sufficient length to serve as a transmembrane region. The remaining 553 amino acids are thought to comprise the large cytoplasmic domain of this isoform. COS cells transfected with this clone expressed a cell associated IL-4 receptor with a molecular weight of 139 kDa. The second class of cDNA exhibited a 5' nucleotide sequence identical to that of the first class through the extracellular region. The sequence encoding the transmembrane domain and the cytoplasmic domains were displaced however, by the insertion of a sequence of 114 nucleic acids 5' to the beginning of the transmembrane region. This insertion results in a shift in the reading frame of the transcript and results in the termination of the coding region before the transmembrane region is translated. Representatives of this class of cDNA were identified in several cDNA libraries of different origin eliminating the concern that the class represented a cloning artifact. COS cells transfected with this cDNA secrete an IL-4 binding protein of molecular weights ranging between 32 and 41 kDa due to different degrees of glycosylation. A naturally occurring soluble IL-4 binding protein has been described in ascites fluid, serum and urine of mice (50). This binding protein appears to be of a similar molecular weight to that of the recombinant soluble IL-4 receptor described above and may

represent the natural soluble form of the IL-4 receptor. This molecule, like the recombinant soluble receptor, blocks the binding of radiolabeled IL-4 to cells.

The IL-7 receptor was cloned by the expression in COS cells of a cDNA library prepared from mRNA isolated from WI 26 cells (19). Complementary DNA clones encoding different isoforms were isolated from this library and one class of cDNA clones encoded a soluble form of the receptor. The gene encoding the human IL-7 receptor was characterized in order to better understand the regulation of IL-7 receptor expression (51). The gene encoding the IL-7 receptor, like those encoding other members of the hematopoietin receptor family (52-55), is composed of eight exons. The exon encoding the transmembrane region (exon 6) can be spliced out resulting in a transcript in which exon 5 and exon 7 are juxtaposed. This event results in a shift in the reading frame and termination of translation within 27 novel amino acid residues (51). This form has been detected in the medium conditioned by the growth WI 26 cells suggesting that it is naturally expressed and secreted. Furthermore, the mRNA encoding the soluble isoform is found in every cell line and tissue in which IL-7 receptor mRNA was detected.

The deletion of the transmembrane region has also been found to occur in the genes which encode the IL-5 receptor (18), GM-CSF receptor (56), and G-CSF receptor (11). The splicing mechanism by which these soluble receptor isoforms are created is reminiscent of the means by which membrane associated and soluble forms of immunoglobulin are generated (57-59).

Soluble cytokine receptors have been identified for IL-2 (60), CSF-1 (61), interferon γ (62), IL-6 (62) and TNF (63). Each of these is thought to arise as a result of proteolytic processing of the membrane associated form of the receptor since no cDNAs encoding soluble forms of these molecules have been identified. It is important to note that these proteins have been purified from biological fluids such as serum or urine emphasizing the notion that these soluble receptors are naturally occurring molecules which bind cytokine ligands with high affinity.

Finally, a soluble TNF binding protein was identified which is encoded in the genome of the Shope fibroma virus (23). This gene contains a nucleotide sequence with significant homology to the p80 subunit of the TNF receptor and does not contain a nucleotide sequence which would encode a transmembrane region of the receptor. The identification of this gene has led to speculation that viral encoded, soluble TNF receptor may contribute to the pathogenicity of this virus in young rabbits.

The numbers of cytokine receptors for which soluble isoforms have been identified has grown significantly over the last year, and the idea that these molecules may play an important role in regulating the actions of cytokines has taken a firm hold. It has thus been of keen interest to determine if soluble receptors might have potential therapeutic value as anti-inflammatory or immunosuppressive agents.

THERAPEUTIC POTENTIAL OF SOLUBLE CYTOKINE RECEPTORS

Soluble IL-1 Receptor - IL-1 is produced by macrophages and is a cytokine with a wide range of different biological activities, among which are included the capacity to mediate many pro-inflammatory events (64, 65). These include the stimulation of the synthesis and secretion of prostaglandin, collagenase, elastase, tumor necrosis factor and other inflammatory proteins. Interleukin-1 can also serve as a chemoattractant for neutrophils and stimulates the expression of neutrophil adhesion molecules on the surface of endothelial cells. Both of these activities serve to promote the inflammatory process. IL-1 is also centrally involved in the generation of antigen specific immune responses, some of which may be detrimental to the host such as in auto immune disease. The identification of antagonists to the activity of IL-1 has thus been the subject of active investigation in the hope that such molecules could be used therapeutically as anti-inflammatory or immuno-suppressive agents. A soluble form of the IL-1 receptor was an obvious candidate to test the value of such an idea.

A soluble form of the murine IL-1 receptor was created by truncating the cDNA just 5' to the region of the gene encoding the putative transmembrane region (Figure 1). The nucleotides encoding the amino-terminal 319 residues of the receptor, were engineered into a mammalian cell expression vector which was then transfected into HeLa cells (66). The medium harvested from these cells contained soluble IL-1 receptor which could be purified by affinity chromatography on an IL-1α column (67). Quantitative analyses of ^{125}I-IL-1α binding to soluble receptor suggested that its ligand binding properties were comparable to those measured for receptor found on intact cells (66, 67). Radiolabeled IL-1α bound to soluble receptors and intact cells with an affinity of 8.4 x

10^9M^{-1} and 6.4 x 10^9M^{-1} respectively. The kinetics of association and dissociation were also similar. Purified soluble IL-1 receptor had the capacity to inhibit the binding of ^{125}I-IL-1 to cells and inhibited IL-1-dependent B cell proliferation, but not IL-4 dependent B cell proliferation (68).

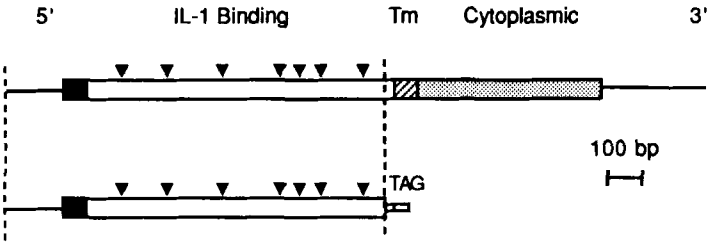


Figure 1. Structure of the truncated IL-1R cDNA. The structure of the full-length cDNA including the leader sequence (*solid bar*), extracellular domain (*open bar*), transmembrane region (*hatched bar*) and cytoplasmic domain (*stippled bar*) are shown. In the soluble construct diagram, the small bar labeled TAG indicates the stop codon after P316.

Soluble IL-1 receptor was tested for its capacity to modulate an immune or inflammatory response in vivo in two models. Firstly, the soluble receptor was administered to BALB/c (H-2^d) mice which had been challenged with irradiated allogeneic spleen cells from C57BL/6 (H-2^b) mice. In the absence of any treatment, the BALB/c mice mount an immune response to the allogeneic cells which can be measured by monitoring the extent of hyperplasia in the lymph nodes draining the site at which allogeneic cells had been administered. In contrast, if soluble IL-1 receptor is administered to the animals at the time of challenge with the allogeneic cells, then minimal hyperplasia of the draining lymph node is observed (69). The inhibition of the node hyperplasia is dependent on the dose of IL-1 receptor administered and can be completely eliminated by the concurrent administration of IL-1 suggesting that the effect is due to the capacity of the soluble receptor to bind IL-1 in this system.

Soluble IL-1 receptor was evaluated for inhibition of graft rejection (69) Soluble IL-1 receptor was administered to adult BALB/c mice into the ears of which heart tissue from C57BL/6 neonate mice had been grafted. The fetal allograft is normally rejected within 12.5 ± 1.0 days. When soluble IL-1 receptor was administered intraperitoneally, the time of rejection was delayed to 18.3 ± 1.5 days. These results suggest that the soluble IL-1 receptor inhibits IL-1 dependent responses in vivo and may be useful in a variety of therapeutic indications including rheumatoid arthritis, multiple sclerosis, organ transplantation, and bone marrow graft versus host disease.

Soluble IL-4 Receptor - IL-4 is produced by T cells and, like IL-1, has range of biological activities (70) which include the stimulation of B cell growth and the maturation of cytotoxic T cells. IL-4 also regulates immunoglobulin heavy chain class expression and is required to sustain an in vivo IgE response. Antagonists to IL-4 action may thus have value in the treatment of allergy, autoimmunity or transplantation. Antibodies directed to IL-4 have been shown to inhibit primary polyclonal and secondary antigen-specific IgE responses in vivo (71) lending credence to this idea. The soluble isoform of the IL-4 receptor has been expressed in mammalian cells and purified by affinity chromatography on IL-4 affinity columns (68, 72). The purified soluble receptor will block the binding of ^{125}I-IL-4 to cells and will inhibit IL-4 specific stimulation of B cells in the presence of anti-surface Ig but not IL-1 specific B cell proliferation. The receptor will also inhibit the capacity of IL-4 to stimulate B cells to produce IgG_1 and IgE suggesting that this receptor may allow the down regulation of IgE production and may thus have value in the potential treatment of allergy.

Soluble TNF Receptor - TNF is a cytokine with a diverse array of biological activities (73, 74). It has many functional similarities to IL-1 and is recognized as a general mediator of inflammation. In particular, TNF is cachectin, the protein produced by macrophages which is responsible for wasting (cachexia) and for toxic shock. The gene encoding the p80 form of TNF receptor has been engineered to terminate the coding region upstream of the transmembrane region and this gene has been successfully expressed in E. coli and mammalian cells. Preliminary experiments with these molecules suggest that they effectively bind TNF with high affinity and hold promise for the

treatment of endotoxin induced toxic shock and are thus likely to be useful for the treatment of other TNF mediated states of inflammation.

Conclusion - The rate at which new cytokine receptor genes have been discovered has been phenomenal. With the elucidation of these genes has come the identification of a number of soluble isoforms of the cytokine receptors which may play a role in regulating the stimulatory effects of cytokines. This prediction has been tested by the engineering and production of the soluble forms of the IL-1 and IL-4 receptors. Both receptor molecules block specific cytokine functions in vitro and are likely to work in vivo to block immune and inflammatory responses. Soluble receptors thus represent a new class of pharmacologic agents that should soon be tested clinically.

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Chapter 24. Amyloidogenesis as a Therapeutic Target in Alzheimer's Disease

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Introduction - Alzheimer's Disease (AD) is a progressive degenerative encephalopathy associated with behavioral disorders, loss of memory and personality, physical debility and ultimately death. It is the most common cause of dementia, afflicting more than 5% of the U.S. population over the age of 65 and nearly 20% of individuals beyond the age of 85 (1).

While AD is certainly an amyloidosis of the brain (see below), it has been shown that neurotransmitter deficits more closely mirror the degree of dementia than the level of amyloidotic deposits in AD brains (2,3). Clinical studies targeting such deficits have been less than satisfying however (4), likely due to a variety of causes including case-to-case heterogeneity of the disease process, differences in diagnostic reliability among clinical centers (5), and complex interactions among various neurotransmitter systems within the brain (6). Since neuronal death is cumulative in AD, neuritic loss and neurotransmitter deficits are expected to increase progressively. Measures of plaque and tangle density must reflect net formation and removal, as suggested in the only published pseudo-time course study of AD (7). The rate of amyloid plaque and neurofibrillary tangle (NFT) formation should therefore plateau as the neurons responsible for the synthesis of their precursors die. While the fundamental genetic or biochemical cause of AD has not yet been defined, amyloid deposition, however removed from the primary event, is a likely participant in the progression of AD-associated neural degeneration. Consequently, amelioration of amyloid-related pathology may prove therapeutically promising since this approach addresses all the various transmitter deficits as well as the neuronal degeneration responsible for dementia. This will be the focus of this chapter.

Alzheimer Amyloid - Amyloid is a generic term referring to extracellular proteinaceous deposits with common morphological properties and staining characteristics. In most cases, the precursors to the amyloid core proteins are disease-specific, raising questions regarding the mechanism of deposition and the differing tissue-specificities among these disorders. These amyloidoses and the properties of the underlying deposits have been reviewed (8,9). Features common to all amyloids include the presence of reticuloendothelial cells such as monocytes, macrophages, or microglia, the acute phase reactant protein serum amyloid P, and tight associations with components of the extracellular matrix (ECM) such as the core protein of the low density basement membrane heparan sulfate proteoglycan (HSPG) and highly sulfated glycosaminoglycans (GAGs) (8,10-13). The latter are perhaps not surprising since amyloid P binds to basement membranes (14), and extracellular amyloid fibrils have been shown to be deposited on basement membranes in a diverse set of senile disorders (15).

Classical AD amyloid plaques are compact spherical extracellular structures, 10-200 μm in diameter, comprised of clusters of swollen dystrophic neurites which contain paired helical filaments, and reactive glial cells intermingled with or surrounding aggregates of 6-10 nm proteinaceous filaments at their cores (16-21). While no single neurotransmitter system is exclusively or completely associated with AD-type pathology (22), core-containing amyloid deposits are found within all regions of AD brains which sustain the greatest degree of neuronal loss and NFT formation. These include the basal forebrain nuclei (23,24), cortical structures (25), and virtually all the interconnecting fiber tracts throughout the limbic axis (25-27). These lesions are found in identical locations in Down's Syndrome (DS). They are also found in lower abundance and with a lower incidence of associated neuritic abnormalities in the brains of aged nondemented individuals (17,28,29). In each of these cases, core-containing plaques which lack neuritic components are more variably found within the parenchymal and leptomeningeal microvasculature (28,30-33).

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In DS, brains from individuals who lived into their fourth or fifth decades generally display all the pathological, degenerative and biochemical changes observed in AD (34,35). The gene encoding the Alzheimer amyloid precursor (AAP) exists on chromosome 21 (36-38) and elevated AAP mRNA has been noted in DS brains (38-40). AAP overproduction may therefore lead or contribute to the array of AD-type abnormalities beginning approximately 30-50 years earlier than in AD. While no evidence exists for gross overexpression of AAP mRNA in AD, certain vulnerable brain regions have shown locally elevated AAP mRNA levels (41-43) as well as enhanced immunostaining for the AAP protein (16,44-46).

Diffuse deposits lacking the central core and tinctorial properties of classical plaques have also been identified by immunostaining with antisera raised to synthetic peptides containing the sequence of the "amyloid plaque core protein" (APCP) (20,29,47,48). These are more numerous than core-containing deposits in AD, DS and control brains (20,21,49,50). As with core-containing deposits, these are also associated with highly sulfated GAGs and the basement membrane HSPG core protein (12,51). Diffuse deposits exhibit a lower incidence of association with neuritic and glial components than core-containing plaques (10,16,17,20,21,35,47,52,53), leading to the suggestion that they are precursors to more "mature," classical plaques (20,47,54,55).

AAP Gene and Transcripts - The isolation and characterization of an approximately 4200 dalton APCP from AD brain (30,31) and the exploitation of its sequence leading to the cloning of corresponding cDNAs (37,40,56,57) have been reviewed (9,21,50,58). At least four mRNAs containing the APCP domain are transcribed from a unique gene by alternative splicing of a primary transcript. These cDNAs encode a signal sequence necessary for secretion, a 150 amino acid domain containing twelve Cys residues, a highly acidic stretch of 95 amino acids, a glycosylation domain so called because of two sites for N-linked carbohydrate attachment, a single hydrophobic transmembrane domain, and a 47 amino acid C-terminal cytoplasmic domain. The first 28 amino acids of the 42-residue APCP are situated N-terminal to the transmembrane domain, placing its fourteen C-terminal residues within the membrane.

The first full-length AAP cDNA cloned encoded a 695 amino acid protein (AAP-695) (37). This AAP mRNA is the most abundant form in brain, and its expression appears to be restricted to neuronal cell types in adults (39,56,59-63). Non-neuronal expression has been noted in early development (40,64) and in cultured type I astrocytes (65). mRNAs encoding additional AAP forms containing 751 (40,56) or 770 residues (57) are expressed in brain and most peripheral tissues (56,59-62). These cDNAs both contain a 168 base pair insert which encodes an active 56-amino acid Kunitz-type serine protease inhibitor (KPI domain) (57,66-72), as predicted from its translated cDNA sequence (40,56,57). AAP-770 contains an additional 19 amino acid domain of unknown function. As expected for genes which express variably spliced mRNAs, these additional domains are contained on distinct exons (57,73). A fourth AAP form, AAP-714 (59,60), is expressed only in trace quantities in brain and peripheral tissues. It contains the same additional 19 amino acids as AAP-770, but lacks the KPI domain. A fifth 3'-truncated variant encodes a 563 amino acid protein which contains the KPI domain but lacks the APCP region (74). Since it is expressed in brain (74,75), it may possess a role in AAP processing, but it cannot be an authentic AAP.

A mutation in the AAP coding sequence has been identified which segregates with the development of AD in two families (76). Such mutations do not account for the majority of AD cases however (76-78). A mutation within the APCP region is associated with a rare hereditary cerebral hemorrhagic amyloidosis (HCHWA-D) (79). Affected individuals develop abundant amyloid deposits within the cerebral and meningeal vasculature, resulting in a high incidence of fatal strokes and subcortical ischemia by early middle age (32,80). Parenchymal amyloid deposits have also been noted in this disorder (32,81), but at lower levels than in AD and without associated neurofibrillary pathology (81). Any potential etiological relationships between HCHWA-D and AD remain unclear.

The gene encoding the AAPs can be induced by nerve growth factor (NGF) (82,83) fibroblast growth factor (83), heparin-binding growth factor 1 (84), phorbol esters (73,82,84), interleukins 1A and 1B (82,84), and retinoic acid (85). Interleukin-mediated AAP induction can be reversed by inhibitors of protein kinase C (84). Splicing of the primary AAP gene transcript has been shown to be influenced by retinoic acid in the neuroblastoma line SH-SY5Y (85) and by NGF in PC12 cells (83), but NGF does not effect splicing in SH-SY5Y cells (85). Germane to this issue, alterations have been reported in the relative steady state levels of the AAP mRNAs in AD brains (39,59,62,75,86-88). These studies have yielded a range of interpretations which is difficult to reconcile, likely due in part to methodological differences, case-to-case heterogeneity in normal aging (39,87), disease stage at death (86,89), and uncontrollable postmortem variables. Notably, correspondence between the levels of AAP mRNAs and protein has been recently questioned

(64). This is a critical issue since it is the relative levels of AAP proteins which are pertinent to AD pathogenesis, rather than those of the corresponding mRNAs. The accumulated data are simply insufficient to determine whether splicing of the primary AAP gene transcript is altered in AD.

AAP Synthesis and Distribution - Numerous studies directed toward identifying the cellular distribution of AAP in brains and cultured cells have employed antipeptide antibodies raised to various AAP segments. Two general observations have been made in sections of AD brain tissue: plaque cores and diffuse deposits are immunostained by antibodies raised to peptides containing the APCP sequence (20,28,29,32,47,49,90-93), while antisera raised to AAP sequences outside the APCP domain stain the coronal region surrounding amyloid cores (28,32,46,91-95). The latter observation raises the possibility that conversion of AAP to APCP occurs in situ. When antipeptide antibodies have been used to investigate structues other than plaques, the results have been more difficult to interpret.

A variety of cells and tissues including megakaryocytes, adrenal gland, and several neuronal types in the central and peripheral nervous systems have been immunostained with more specific antisera raised to recombinant AAP (96). In sections of AD brain tissue, these antisera stain normal and dystrophic neurons and neurites, neuritic lysosomal bodies and coronal regions surrounding amyloid plaques (16,28,96). These observations are consistent with a subset of reports which employed antipeptide antisera, showing staining of neuronal perikarya (97,98), dystrophic neurites surrounding amyloid cores (45,46), perinuclear regions, secondary lysosomes (44,95,99) and lipofuscin (49,90).

Immunostaining of some glial cells has been reported in AD brain sections (65,96), but well below the levels within dystrophic neurites surrounding plaques (16). Astrocytic staining has been noted proximal to intrahippocampal injections of colchicine or excitotoxins in rats. While this suggests that neuronal damage may precede astrocytic AAP expression (97), the relevance of these types of lesions to AD is uncertain. It remains to be determined whether glial staining is due to endogenous expression or to phagocytic uptake in AD, particularly since AAP immunostaining is not a uniform glial characteristic within sections of normal and AD brain tissue (16). With one exception (100), immunostaining of plasma membrane has been unconvincing (98,99), contrary to expectations based on the sequence of the AAP cDNA (37).

Circulating (32,58,101,102) and glial (13,97) precursors have been proposed as non-neuronal sources for parenchymal amyloid. While AAP is found in platelets (103-105), it is also expressed in neurons (39,41-43,61,88,89,106,107), glia (43,59,65), vascular endothelial (43) and vascular smooth muscle cells (108). No a priori need therefore exists to postulate a single source for vascular and parenchymal amyloid. Indeed, in situ hybridization studies suggest that the distribution of amyloid deposits within cortical and subcortical structures is afforded by the neuritic projections of neurons which synthesize the greatest quantities of AAP mRNA (39,41-43,61,88,89,106,107). This has been well shown within the hippocampal formation in which neurons either giving rise to the perforant pathway or projecting to its termination zone contain abundant AAP mRNA (42,43,61,106,107). In AD, these neurons develop NFT's, and their axons project to regions heavily endowed with amyloid plaques (26). A similar relationship between tangles and plaques has been suggested within cortical and subcortical structures (25). These observations are consistent with recent reports that AAP undergoes anterograde axonal transport (109) and accumulates at neuritic terminals proximal to amyloid deposits in AD (16,28,44,91). This specificity of amyloid deposition could not be provided either by a vascular or a glial source of precursor. Recent reports indicate that spatial relationships do not uniformly exist between vessels and parenchymal deposits (33,48,110), despite earlier indications to the contrary (101,111). Dystrophic neurites immunostained for AAP are found proximal to diffuse deposits which lack both capillary (48) and glial involvement (16,20), strongly arguing against these non-neuronal sources if diffuse deposits are really "preamyloid." It is therefore more likely that vascular and parenchymal amyloid are derived from independent sources (110,111), and that parenchymal amyloid originates from neuronally-produced AAP. A role for glial-derived AAP remains an open question.

AAP Protein -- Processing and Function - AAP proteins are N-glycosylated (63,112,113), O-glycosylated (63,113,114), tyrosine-sulfated (63,113,114) and phosphorylated (113). It has been suggested that the AAPs may span a membrane (37,115), function as a cell-surface receptor (37) or be involved in cell contact (115). Studies employing cell-free translation of full length cDNAs have shown that they are integral membrane proteins (112), but the other proposed functions have not yet been confirmed. Despite their calculated molecular weights (78.6-86.9 kDa), native AAP's show apparent molecular weights ranging from 95-150 kDa in SDS-polyacrylamide gels (95,99,109,114,116-120). This is presumably due to reduced SDS binding by the acidic domain of

the protein (112) since glycosylation accounts for only part of these discrepancies (63,112-114), and since AAP's produced by recombinant expression in E. coli (63,68), insect cells (68) and mammalian cells (95,113,121) all exhibit sizes similar to endogenous mammalian AAP's (95,116,118,119,122).

One or both of the KPI-containing AAP forms appeared in the literature in 1987 as the serine protease inhibitor "protease nexin II" (120). This was confirmed in a study utilizing recombinant AAP expressed in human 293 cells, showing that the sizes of secreted AAP-751 and nexin II were identical, as were their affinities for dextran sulfate and trypsin (123). Nexin II was later shown to be stored in platelet alpha-granules and secreted upon degranulation (103-105), suggesting that it may have a role in wound repair, coagulation and inflammation. The KPI domain and the AAP forms in which it is contained inhibit a limited spectrum of serine proteases. They are potent inhibitors of trypsin, chymotrypsin, and coagulation factor XIa (67-72). They are weak to moderate inhibitors of plasmin, gamma-NGF, mouse EGF binding protein, kallikreins, the mast cell proteases tryptase and chymase, leukocyte elastase, and factor Xa (66-70). They do not inhibit cathepsin G, factors VII_a or XII_a, serum complement or complement factors C1s and C1r, papain, acetylcholinesterase, tissue plasminogen activator, clipsin, urokinase or thrombin (66-71). Natural cognate proteases have not yet been identified in brain.

While other suggested functions for the AAP's remain conjectural (83,119,122,124-129), the relevance of AAP function to AD pathogenesis is unclear, as is the relationship between precursor function and amyloid deposition in any amyloidosis. AD-type amyloidogenesis may merely be due to altered AAP processing leading to the accumulation of amyloidogenic peptides within vulnerable brain regions. Accordingly, short peptides containing fragments of the APCP sequence are intrinsically amyloidogenic (130,131), and the C-terminal 100 amino acid AAP segment, which aggregates when free of association with membranes, is reduced to an APCP-sized fragment when digested with proteinase K (112).

Peptides containing partial APCP sequences exhibit neurotoxic, neurotrophic and neuronal survival activities in cultured cell systems (124,125,128,129). These peptides can sensitize primary rat cortical neurons to excitotoxic damage (127). Since aggregates of APCP should provide a limited source of soluble peptide, these observations may explain in part the seemingly contradictory phenomena of neuritic outgrowth (132) and degeneration proximal to amyloid deposits in AD brains (16,20,28,44-46,96). These effects may depend on the local concentration of soluble peptide (125-129) and the developmental stage of the affected cells (125-127).

Soluble secreted AAP derivatives corresponding to both AAP-695 and to KPI-containing forms have been identified in brain, cerebrospinal fluid and in media conditioned by various cell types (63,71,83,103,104,133,134). These truncated forms extend from the same N-terminus as the full length AAPs to a C-terminal cleavage site within or near the APCP domain. A similar cleavage has also been reported in recombinant systems expressing full length AAP (63,68,113,121,135). It is important to understand the specificity of this cleavage since it may impact amyloidogenic mechanisms in AD. In the first of two studies which directly addressed this issue, various AAP segments were fused to reporter genes and expressed in several transformed cell lines to determine the minimal sequence around the APCP domain necessary for cleavage (136). In the second, full length AAP was expressed in human 293 cells and the two fragments generated by the so-called "secretase" activity were purified from cell lysates and conditioned media. The C-terminus of the secreted segment and the N-terminus of the 10-14 kDa cell-associated fragment were then sequenced (135). Both studies concluded that the "normal" secretase cleavage occurred within the APCP domain, precluding its role in amyloid formation. If this is the only "normal" cleavage which occurs in brain, these studies implicate both age- and disease-related secretase deficiencies since APCP does occur in AD, DS and normal aging.

Similar studies have not yet been performed in cells of CNS or neuronal lineage. A critical question thus concerns the use of the term "normal," since several studies suggest that the normal situation may not be so clear-cut. In addition to bands on Western blots corresponding to the full length AAPs and their secretase-generated fragments, several intermediate-sized proteins have been noted in brain extracts (44-46,49,64,90,94,95,133,137), CSF (133,138), cultured cells (82,100,118) and recombinant systems (113,121). Full length AAP expressed in CV-1 cells as vaccinia virus recombinants exhibited 12- and 16-kDa cell-associated C-terminal fragments (121). The 16 kDa protein comigrated with a C-terminal AAP segment expressed in the same recombinant system which contained the entire APCP region. PC12 cells generate 15- and 19 kDa C-terminal peptides, induced either by protein kinase C or by inhibition of protein phosphatases 1 and 2A

(118). PC12 cells also release elevated quantities of a 60 kDa C-terminal AAP-fragment containing the entire APCP domain when stressed by culturing in serum free media, or when cultured in the presence of a membrane damaging reagent or lysosomal inhibitors (82). It has also been suggested that activated platelets release both C-terminally truncated and full length AAP forms (104).

Several points can be raised based on these observations: 1) AAP cleavage may be more complex than suggested by studies in cultured transformed cells (135,136). These studies characterized a predominant cleavage, but did not address less frequent cleavages which may account for a slowly progressive amyloidosis. The suggested role of AAP in cell adhesion may be relevant in this regard (83,100,115), since altered cell adhesion is known to accompany cellular transformation (139). 2) We do not know how the AAPs are processed in normal or AD brain. In this context, results consistent with disease- and tissue-specific AAP processing may be pertinent (96,137,140), as is a report of a glioma cell line which apparently lacks secretase activity (100). 3) Neuronal injury may result in augmented release of amyloidogenic AAP fragments (82). It is nevertheless important to purify and characterize the AAP secretase, and determine whether (and to what degree) its activity might be altered or circumvented in AD and DS brains.

AD Amyloidogenesis - The presence of complement proteins (20,52) and lysosomal proteases (141,142) within plaque cores suggests that AD amyloid is subject to proteolytic attack. In addition to secretase deficiencies, other alterations in proteolytic processing of the AAPs may be etiologically significant to amyloid formation (50,143,144). In this regard, the acute phase reactant alpha$_1$-antichymotrypsin is associated with AD-type amyloid deposits (143-145), but not with other cerebral amyloids (145). Since astrocytic expression of this serine protease inhibitor appears to be induced surrounding damaged tissue in a variety of neurological disorders (144), it is not known whether these interactions are specifically involved in AD amyloidogenesis or a secondary effect of affinities between alpha$_1$-antichymotrypsin and the APCP or its precursor.

It has been postulated that APCP must be proteolytically released from the AAP in advance of amyloid fibril formation (102). This says little about the nature of the initial deposits which nucleate the amyloid plaque. The possibility that conversion of AAP to APCP occurs in situ raises the prospect of a general proteolytic response to nonfibrillar deposits of AAP, leaving an APCP "residue" which is difficult to metabolize further. A net elevation in the rate of prefibrillogenic AAP deposition might therefore overwhelm the brain's ability to properly catabolize incipient deposits. It is obviously critical to understand the interactions of intact AAPs with other known components of AD-type amyloid deposits, as well as the mechanisms by which AAP's containing the intact APCP domain are released from their cells of origin.

Extremely high affinities which exist between the AAPs and components of the ECM may be enlightening in this regard (146). The low density basement membrane form of the HSPG purified from the Engelbreth-Holm-Swarm tumor (147) binds to recombinant AAPs in a concentration-dependent and saturable fashion, consistent with a simple equilibrium involving a single AAP binding site. This is the same HSPG which has been shown to be an integral component of AD amyloid fibrils (51). The binding of HSPG to AAP-695 exhibits a Kd of approximately 0.9 nM, while binding to the forms which contain the KPI domain exhibit Kd's approximately 10-fold higher. This is particularly intriguing in light of the neuronal specificity of AAP-695 expression (39,56,59-63), potentially explaining the brain-specificity of AD-type amyloidosis (96). This further suggests that it may be possible to interfere with these higher affinity interactions in brain without altering peripheral associations involving the KPI-containing AAP forms (146). Similar affinities exist between the AAP's and laminin (146). Interactions between AAP and collagen type IV have also been reported (148), as has immunostaining of core-containing plaques with antibodies to fibronectin (149).

These observations raise the possibility that AAP-ECM interactions may provide a nidus for amyloid formation. The association of heparan sulfate with diffuse deposits (51), as well as AAP-ECM interactions in cultured cells (150) are consistent with this suggestion. A normal equilibrium between AAP production, secretion, ECM-binding and catabolism could be disrupted in a variety of ways including overexpression of the AAP as in Down's Syndrome, or altered production of enzymes or structural proteins involved in these interactions in AD.

It is interesting in this regard that dense immunostaining for HLA-DR has been observed over microglia both within AD plaque cores (11,16) and within diffuse deposits (10). Classical complement pathway antigens have been observed within AD amyloid deposits (52,151-153), as has the membrane-attack complex C5b-9 on surrounding neuritic terminals (52). Since the

functions of peripheral macrophages are executed by microglia within the brain (18,19,154), an inflammatory response to inappropriate ECM-AAP structures could ultimately result in the fragmentation of these complexes, initiated through a local microglial-mediated complement cascade (for review, see 155). This type of lytic attack on neuritic terminals would result in the release of neuritic components, accounting for the presence of active lysosomal proteases within plaque cores (141,142). Consistent with a role for complement attack in plaque-associated neuritic dystrophy, complement proteins are not found within amyloid deposits which lack a neurofibrillary component, i.e. those associated with prion-based spongiform encephalopathies or cerebellar "preamyloid" in AD (53). Neuritic degeneration initiated by the attack complex may therefore explain the presence of plaque-associated NFT's in AD, and the absence of NFT's in diffuse deposits and (but for one exceptional report (156)) in association with amyloid deposits in prion-based disorders (157). This scenario admittedly places tangle formation secondary to amyloid deposition. While unproven, it is consistent with accumulating evidence that amyloid deposition precedes tangle formation (28,29,34,50,54,55,158,159).

These interactions could all lead to the formation of amyloidotic fragments of ECM-bound AAP which are resistant to further proteolysis, generating compacted plaque cores, neuritic dystrophy, and trophic/toxic AAP fragments (125-129). This model does not require alterations in secretase activity to explain the formation of intact APCP since degenerating neurites could provide a source of full length AAP. It does require a relationship between inflammatory responses and a threshold level of AAP-ECM complexes to account for the distribution of amyloid deposits within the brain, since AD pathology is not a general feature of brain inflammation. While the mechanism of plaque-associated C1 activation has not been clarified (151-153), it is interesting that non-immunological complement activation can be initiated by plasmin (155). Since KPI-containing AAP forms are moderately strong inhibitors of plasmin (66-70) and presumably other plasmin-like proteases, the ability of AAPs to inhibit non-immunological complement activation may be specifically compromised in brain due to the more abundant expression of AAP-695 in comparison with the exclusive expression of KPI-containing AAP forms in the periphery.

Therapeutic Approaches - Events involved in the cycle of amyloid development and removal include nucleation of the deposits, the growth of the nucleated mass, proteolytic processing of the precursor and eventual dissolution, either by phagocytic processes or direct proteolytic attack. Each provides a potential target for drug intervention.

Proteolytic processing of the AAP appears to be a prime therapeutic target. The application of enzyme-specific inhibitors might be a valid therapeutic approach if deposition is enhanced by inappropriate release of amyloidotic fragments prior to normal catabolism of the AAP. If so, even partial inhibition of the responsible protease might achieve the desired goal. If deposition results from the inhibition of a normally active catabolic protease, by the KPI domain of the AAP for example, interference with the formation of the enzyme-inhibitor complex appears attractive theoretically. This approach would be inexpedient however, because of the ubiquitous nature of trypsin inhibitors. The risks of such approaches will depend on the range of substrates acted upon by the target proteases. These are speculative and long-range approaches since we do not know the relevance of either proteolytic induction or inhibition, nor do we know the appropriate enzymes.

Therapeutic removal of existing deposits would be difficult due to their relative intractability to proteolytic digestion, despite the presence of peptide bonds within the APCP which are normally susceptible to enzymatic attack (see above). This approach will necessitate the development of methods to disrupt the amyloid fibrils, granting sufficient access to the ambient degradative enzymes, tipping the equilibrium away from net deposition. This is a speculative and long-term goal.

If the nucleation and growth of amyloid deposits involve specific interactions between segments of the AAP and the ECM (see above), the formation of these complexes could be mitigated by small molecules mimicking the "active site" of either the AAP or the ECM. In this case, the inhibition of amyloid nucleation on the ECM would slow the progressive amyloidosis, better enabling the net clearance of existing deposits by normal catabolic processes.

Finally, an analogy between AD and inflammatory disorders may be appropriate since both are characterized by release of lysosomal enzymes, induction of acute phase reactant proteins, and formation of amyloid deposits in effected tissues. It is interesting in this context that AAP can bind TGF-beta produced by glioblastoma cells (117), particularly in light of effects of TGF-beta on the expression of ECM components (160,161). Additional activities of TGF-beta which may prove relevant to brain amyloidosis include its role as a chemotactic ligand for monocytes (162), and its activation of monocytes to secrete interleukin-1 (161,162), known to be elevated within glial cells in

AD and DS brain (163) and to induce AAP gene expression (82,84). It is therefore extremely intriguing that the incidence of AD in rheumatoid arthritis patients undergoing anti-inflammatory therapy is significantly below that of the general population (164). While this observation must be considered anecdotal at this time, it is clearly an important point to study in detail since modulation of inflammatory responses may provide a reasonable path toward therapeutic approaches to AD.

Notably, each of these scenarios lacks an understanding of primary etiological events leading to the proposed pathological cascade. While this is an ultimate step toward maximally effective therapy, it must be recognized that it is not necessary to cure a disorder in order to affect treatment, as witnessed by insulin administration for diabetes and l-dopa for Parkinson's Disease. Treatments which either delay the age of onset or slow the rate of disease progression will significantly reduce the morbidity of AD.

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Chapter 25. Bacterial Adhesins

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Introduction - Adhesins are proteins located on the surface of bacteria that mediate their attachment to specific substrates as a prelude to colonization. Substrates may be inanimate surfaces, eucaryotic cells or other bacteria. The relatively specific nature of adhesin interactions differentiate their mode of action from other surface molecules that participate in comparatively weak, non-specific hydrophobic or electrostatic reactions. Two general types of adhesive interactions have been described. Lectin-like adhesins recognize specific sugar moieties in receptor molecules (i.e. glycolipids, glycoproteins or polysaccharides) while the second group possess binding sites that recognize non-carbohydrate constituents, i.e. protein-protein interactions. The former group of interactions are similar, if not identical, to those observed with plant lectins (1, 2). In general, many bacterial lectin-like adhesins recognize receptor molecules containing galactosides, aminogalactosides, n-acetylated amino galactosides, methylgalactosides, combinations thereof and neuraminic acid. Glucose and mannose appear to be used less frequently as receptor sugars. The carbohydrate binding domains of some phytoagglutinins have been defined physically and chemically while their physiological function is poorly understood. Paradoxically, the function of many bacterial adhesins is well established, but the structures of their binding domains have not been well characterized.

With few exceptions, a microorganism cannot be an effective pathogen unless it adheres to and subsequently reproduces within a host. Thus, the central role played by adhesins in disease processes has elevated these protein molecules to the status of virulence factors and currently they are used to define a bacterium's pathogenic potential (3). Although pathogenic bacteria have been studied extensively over the past century, the nature and mode of action of the molecules that contribute to their invasiveness were not fully understood. However, investigations into the functional and structural features of adhesins, toxins and ancillary invasive molecules have begun to remedy this deficiency. Moreover, studies of pathogens have had the salutary effect of stimulating investigations into the attachment of saprophytic bacteria.

By definition, hemagglutinins are adhesins (4) and most of the adhesive proteins discussed in this review possess the ability to agglutinate human or animal erythrocytes. However, a comprehensive discussion of these proteins is neither possible nor necessarily germane to the ensuing discussion since the in vivo function of many bacterial hemagglutinins has not been established yet. Therefore, this review will be restricted to a description of a select number of bacterial proteins that possess specific attachment functions relevant to colonization.

Adhesin Topology - Bacterial adhesins occur in one of two general configurations on a cells' surface. Non-fimbrial adhesins are generally anchored in the outer membrane or cell wall (peptidoglycan layer) of bacteria (5, 6). Fimbrial-associated adhesins are associated with hair-like structures (fimbriae or pili) that extend away from the cell surface (7). The latter are believed to provide an advantage for attachment by positioning adhesins such that the electrostatic repulsion which is generated between the interacting surfaces (bacteria and substrate) is reduced or nullified (8). Figure 1 depicts the arrangement of adhesins on the surfaces of bacteria and a pictorial model for initiating colonization. Example A represents a microbe with adhesins on appendages, i.e. pili or fimbriae; example B is a bacterium with its adhesins incorporated into the cell wall (Gram positive bacteria) or outer membrane (Gram negative bacteria); and C shows a primary colonizing bacterium serving as a bridge for a secondary colonizer. Specific examples of the three themes are presented below.

Prototypic *Escherichia coli* Adhesins- As a group, the uro- and enteropathogenic strains of *E. coli* possess the best characterized microbial adhesive proteins. It has become common practice to distinguish the various *E. coli* adhesins according to the carbohydrate receptors each recognizes.

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For example, the lectin-like molecules are identified by the sugar(s) in the carbohydrate receptor that interact with the adhesin's binding site or the sugar that most effectively inhibits attachment. Thus, the type 1, *pap* (P blood group) and S fimbrial adhesins are also known as the α-mannose, digalactose and sialic acid adhesins, respectively (9, 10); where the receptor is unknown, the term "X" adhesin has been used (11). This mode of identifying adhesins has been extended to other groups of bacteria.

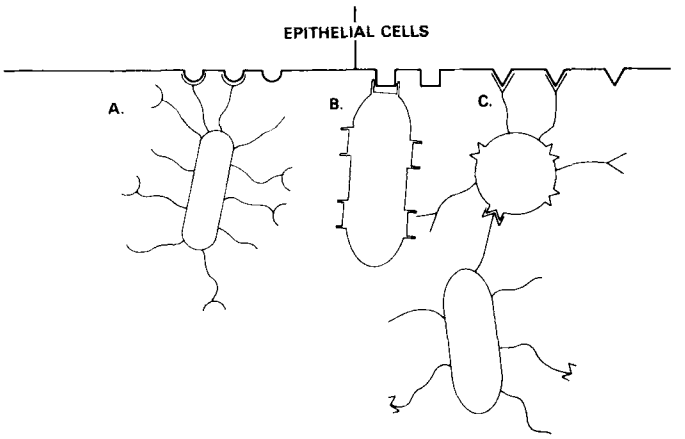


Figure 1. Modes of attachment of bacteria to host cells mediated by specific adhesin and receptor interactions. Example A represents a microbe with adhesins on appendages such as pili or fimbriae; Example B is a bacterium with its adhesins incorporated into the cell wall; and C is a primary colonizing bacterium serving as a bridge for a secondary colonizer.

Until recently, it was believed that the ability of fimbriae or pili to interact with human tissue receptors was a property of the structure itself. This paradigm had to be modified in 1985 when it was demonstrated that *E. coli* continued to synthesize the *pap* adhesin after synthesis of the fimbrial structural gene product had been abrogated by transposon insertional mutagenesis (12). Subsequently, a spate of investigations of the type 1, P and S fimbriae revealed that the fimbrial subunit and adhesin were two distinct proteins (13 - 17). Generally, fimbriae from *E. coli* and related bacteria are composed of 16 to 18 kDa subunits while the adhesin monomers are roughly 30 kDa polypeptides (13, 18, 19). In contrast to many bacterial proteins (e.g., the fimbrial subunit), adhesins are found in relatively low numbers and have been difficult to purify in quantity by conventional techniques. The ratio of the two proteins, adhesin to fimbrial subunit, has been estimated to be between 1:60 and 1:1,000 which led to the former being called a "minor component" (19 - 21).

Much of the available information concerning the properties of adhesins has been deduced from their amino acid sequence obtained by translation of the gene nucleotide sequence or mutational analyses. The latter technique was used to establish that the receptor binding site on the *pap* adhesin was located in the amino terminal portion of the molecule (22). Similar experiments revealed that an alteration of lysine-116 or arginine-118 of the sialic acid (S) adhesin resulted in a partial or complete loss, respectively, of hemagglutinating activity (23). The precise location of the *pap* adhesin molecule on the P fimbriae is being debated currently. One group has reported that the pap adhesin is found exclusively on the tip of the fimbriae (24, 25) while others presented

evidence showing that the adhesin molecule is not restricted to the tip but is also found along the stem of the structure (26 ,27). Both groups agree that the adhesin is not found on every fimbriae. The mannose-sensitive adhesin on type 1 fimbriae is found both at the tip and along the stem of the structure (15, 28).

Within the last three years, the respective fimbrial subunit and adhesin genes were shown to be parts of significantly larger complex operons containing as many as 11 genes (13, 15, 18, 29, 30). The operons contain genes that code for a membrane channel protein that fixes the site of fimbriae assembly, a protein (chaperone) which guides the fimbrial subunits and adhesins through the cytoplasmic membrane and accessory proteins that are associated with the adhesin at the point of attachment to the fimbriae (28, 31). X-ray crystallography of the purified chaparone indicated that the protein was topologically similar to immunoglobulin but shared no primary sequence homology with the latter (32). However, 26 percent of the amino acid sequence of the bacterial protein was identical to that of Leu-1 human lymphocyte differentiation antigen, CD5. The significance of the sequence homology, if any, is unknown.

Uropathogenic strains of *E. coli* have been isolated that express non-fimbrial (or afimbrial) adhesins (5, 6, 33). Like the fimbrial associated adhesins, the two proteins described below are also hemagglutinins and, in one study, it was demonstrated that the gene coding the adhesin is part of a large transcriptional unit containing at least five genes (34). Both adhesins are smaller than their fimbria-associated counterparts with molecular masses between 16 and 21 kDa. The biochemical nature of the receptors of the two adhesins are unknown and they may not be lectin-like proteins.

Strains of *E. coli* possess the genetic information to synthesize more than one type of fimbriae (and adhesin) and, within a population, cells bearing either P or type 1 fimbriae (and their respective adhesins) are readily detected (34). An exception to the generalization that each type of fimbriae bears only one type of adhesin appeared recently when a second *pap* operon was discovered (35). When translated, the second operon synthesized the expected P fimbrial gene product, but produced a different type of adhesin. The new operon was designated *psr* (*pap* related sequence) and its adhesin recognized a carbohydrate receptor containing the amino sugars GalNAc α(1-3) GalNAc. A comprehensive comparison of the two adhesins may provide clues to the evolution of the two, functionally dissimilar adhesive proteins.

Reports indicate that tactile signals generated upon physical contact of enteric bacteria with host epithelia trigger the expression of bacterial genes encoding "invasive proteins" which subsequently permit the pathogenic bacteria to penetrate the host cell (3, 36, 37). Whether a contact type of signalling mechanism is responsible for the synthesis of a particular type of fimbriae and adhesin has not been proven. It seems likely that a constitutively synthesized, adhesin-like protein is essential to establish and maintain initial cell-to-cell contact for successful signal transduction. Global regulatory signals modulating the expression of the fimbrial-adhesin operons are currently being defined. A gene regulating temperature-sensitive expression of the *pap* operon has been cloned and characterized; the gene product is responsible for reducing transcription of the operon at temperatures below 37^{o} (38). Strains of *E. coli* bearing the S fimbrial adhesins respond to growth on a solid surface by increasing the synthesis of the adhesive protein; the mechanism of action is unknown (39).

Pseudomonas aeruginosa Adhesins - *P. aeruginosa* is an opportunistic pathogen of patients with cystic fibrosis. This microorganism provides an interesting counterpoint to the "minor component" adhesins found among enteric and other bacteria. A compelling body of evidence exists supporting the notion that the adhesive activity mediating attachment to epithelial cells resides in the 15 kDa pili monomer (pilin molecule). Purified pili and peptide fragments derived from pili competed with intact bacterial cells for binding sites on buccal and tracheal epithelial cells (40). A synthetic peptide identical in sequence to adhesin residues 128 through 144 with reduced cysteines at positions 129 and 142 competed effectively with pili in binding to epithelial cells. These data indicated that the attachment site was in the carboxyl terminus of the pilin molecule (41). This notion was reinforced when a group of eight antisera were prepared against peptides representing predicted surface regions within the pilin molecule. Only the antiserum prepared against the cysteine-bridged carboxyl terminus peptide blocked adherence of the pili to buccal epithelial cells (42). Intact pili were used to identify the adhesin receptor on buccal epithelial cells. The surface glycoproteins were resolved by denaturing electrophoresis, blotted onto nitrocellulose and identified immunologically using a pili/anti-pili staining system. Pili bound to five glycoproteins ranging in size from 40 to 82 kDa. FAb fragments of a monoclonal antibody specific for a region near or at the adhesin binding site of the pilin polypeptide blocked adherence of pili to the glycoproteins.

The adhesin appears to be lectin-like because periodate treatment of the immunoblots prior to adding pili inhibits attachment as does the presence of L-fucose and sialic acid (43).

Bordetella pertussis Filamentous Hemagglutinin - The causative organism of whooping cough, *B. pertussis*, synthesizes an adhesive structure, filamentous hemagglutinin (FHA), which is distinguished from its fimbriae on the basis of size and function. FHA has been purified and characterized and the gene encoding its structure has been cloned and sequenced (44 - 46). It is a rod-shaped structure 2 nm by 40 - 100 nm that migrates as a single protein band in native, acidic polyacrylamide gel electrophoresis (PAGE). Denaturing PAGE resolves the protein into three major bands with M_r of 200 kDa, 130 kDa and 100 kDa. The three bands are immunologically related and the 200 kDa polypeptide (some reports cite the M_r as 220 kDa) is the active form of the adhesin. Taking advantage of FHA defective *B. pertussis* mutants and a source of purified FHA, the adhesin's role in adherence to the ciliate epithelia of the throat has been documented with a number of different in vitro assays (47, 48). The active site of the adhesin contains a sequence of arg-asp-gly (RDG region) (47) which recognizes a class of protein molecules known as integrins (49). These include connective tissue proteins such as collagen and fibronectin.

Mycoplasma Adhesins - *Mycoplasma pneumoniae* adheres to, but does not invade, lung tissue producing a recalcitrant form of pneumonia. The P1 adhesin, a 165 kDa polypeptide intercalated in the organism's membrane, was identified immunologically with MAbs that specifically inhibit attachment in a chicken erythrocyte cytoadherence system (50, 51). Immune electron microscopy established that the adhesin molecules were concentrated at the tip of the flask shaped bacterium in an area designated as the tip organelle (52, 53). A *Mycoplasma genitalium* membrane-associated 140 kDa protein shared epitopes with the 165 kDa adhesin. The 140 kDa protein is thought to participate in attachment of *M. genitalium* to host tissue (54). The genes coding the two adhesins were cloned and sequenced. A comparison of the sequences revealed conserved areas of homology which were believed to represent the cross-reacting epitopes (54). Studies with radiolabeled *M. pneumoniae* cells and purified glycoconjugates disclosed that the adhesins recognized 2-3 linked sialic acid moieties on glycoproteins and certain sulfated neutral glycolipids (55, 56). A host-borne receptor for the P1 adhesin was recently isolated from MRC-5 human lung fibroblasts (57). Both the isolated 100 kDa glycoprotein receptor and anti-receptor serum inhibited attachment of *M. pneumoniae* to MCR-5 fibroblasts. Surprisingly, the receptor interacted with 32 kDa, 90 kDa and 139 kDa proteins on *P. pnuemoniae, P. genitalium* and *Mycoplasma gallisepticum*, respectively. These data suggest that the two former mycoplasmas may possess more than one type of adhesin on their surface.

Adhesins of Oral Bacteria - Dental plaque deposits represent a complex microbial community containing scores of Gram positive and Gram negative bacterial species (58, 59). The diversity of the oral community makes plaque a truly novel ecosystem and stands in marked contrast to "blooms" of a single type of bacterium seen in other environments. Plaque accretion is the result of highly specific interactions between the genetically diverse members of this unique bacterial consortium (60, 61). The primary colonizing bacteria (certain streptococci and actinomyces) possess adhesins which interact with the salivary proteins or glycoproteins that coat the tooth surface (62, 63). Subsequently, other oral bacteria bearing adhesins which recognize receptors on the primary colonizers attach to the latter adding to the size and diversity of the bacterial community. A number of Gram negative oral bacteria are secondary colonizers of plaque. Among these secondary colonizers, species of *Prevotella, Porphyromonas* and *Capnocytophaga* have been implicated as causative agents of periodontal disease. They also can be opportunistic pathogens affecting other tissues of the host (64, 65, 66). Thus, understanding how these secondary colonizers attach at the gingival crevice has become a matter of importance to dental scientists. Figure 2 depicts several aggregations among oral bacteria that are readily demonstrated in vitro.

As was the case with the enteric bacteria, extensive preliminary surveys have established that both protein-protein and lectin-like interactions contribute to the deposition of bacteria in the oral habitat (67). Similarly, adhesins may be situated on fimbriae or intercalated into the outer membrane (or cell wall) of the adhering bacterium. Many of the lectin-like reactions can be inhibited or reversed by galactosides, aminogalactosides or other sugars (67, 68). Screening for these types of cell-to-cell interactions is relatively simple; suspensions of the two types of bacteria are mixed, if between them they possess a complementary set of adhesin and receptor molecules, visible aggregates are produced (69).

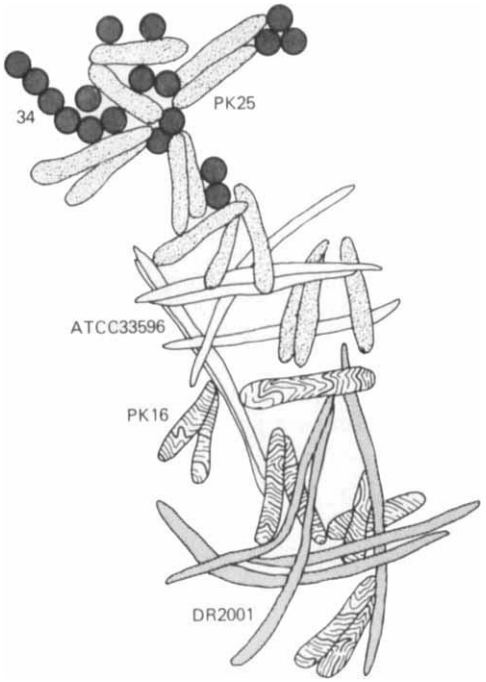


Figure 2. Some naturally occurring cell-to-cell interactions among oral bacteria that simulate the accretion of plaque. Strain 34 (*S. oralis*) and PK 25 (*Actinomyces israelii*) are primary colonizers that attach to tooth pellicle (a salivary coating). The secondary colonizing bacteria *C. ochracea* (ATCC 33596) and *C. gingivalis* (DR2001) interact via the "bridging" organism *A. israelii* (PK16).

Prevotella (formerly Bacteroides) Adhesins - *Prevotella loescheii* attachs to two members of the plaque flora, a primary colonizer *Streptococcus oralis* strain 34 and *Actinomyces israelii* strain PK14 (70). The adhesin mediating the former interaction is a lectin-like protein which recognizes N-acetylated galactosamine residues while the latter appears to be a protein-protein reaction. Sugar inhibition studies and experiments with naturally occurring adhesin-negative mutants indicated that the streptococcal-specific adhesin was also a hemagglutinin capable of reacting with a large number of neuraminidase-treated mammalian erythrocytes (71).

Because virtually nothing is known about the organization of the bacteroides genome and comparatively few recombinant DNA techniques have been developed for these bacteria, a different approach was required for the identification and characterization of the *P. loescheii* adhesins. Anti-*P. loescheii* (wild type) polyclonal antiserum adsorbed with an adhesin negative mutant, retained the ability to inhibit the interactions between *P. loescheii* and its two partner strains indicating that IgG specific for the adhesins remained in the serum. This fact made it possible to adopt an immunological approach to identify and purify the adhesive proteins (72).

Specific anti-adhesin monoclonal antibodies (MAb) were identified by testing hybridoma supernatants primarily for their ability to inhibit the cell-to-cell interactions (73). MAb IgG and Fab fragments proved to be exquisitely potent inhibitors of these reactions (effective in the range of ng of IgG protein) and were used in immunoblot analyses to establish that the streptococcal-specific and actinomyces-specific adhesins consisted of 75 kDa and 43 kDa subunits, respectively (74). Both adhesins are significantly larger than the enteric adhesins. MAbs labeled with ^{125}I were used in antibody saturation studies with intact *P. loescheii* cells to determine the theoretical maximum number of adhesin molecules per cell. Values of 300 and 500 molecules per cell were obtained for the actinomyces-specific and streptococcus-specific adhesins. respectively (74). Gold particles coated with the appropriate MAbs were used to locate each of the adhesins on the surface of the bacteroides cells. Immunoelectron microscopy showed both types of adhesins were associated

with the distal portion of the organism's fimbriae (71, 75). Their arrangement was not as precise as that reported for the enteric bacteria, rather gold particles were found singly, in pairs or in small clusters along the distal portion of the fimbriae and, not all fimbriae bore adhesins. Furthermore, the gold particles were not restricted to the tip of the structure. In other experiments, it was shown that each cell in the population possessed both types of adhesin (75).

The 75 kDa subunit streptococcal-specific adhesin of *P. loescheii* was the one of the first adhesins from an oral Gram negative bacterium to be isolated and characterized. Purification was accomplished by affinity chromatography using anti-streptococcal adhesin specific MAbs conjugated to Sepharose 4B (76). The adhesin is a basic protein with a pI of between 8.0 and 8.4 and a native molecular mass of the adhesin was 450 kDa indicating that the adhesin exists as a hexamer. The purified adhesin was maintained at a pH of 4.5 to 5 because the protein began to aggregate as the pH of the solution was increased towards its pI; at its pI, the protein became insoluble and denatured irreversibly. At a pH between 5.8 and 6.8 the adhesin appeared to pass through a transition state forming soluble aggregates. The purified adhesin retained its binding capabilities, but its ability to affect adherence was dependent on its state of aggregation. Thus, when preincubated with *S. oralis* 34 at a slightly acidic pH, single adhesin molecules bound to the streptococcal receptor and inhibited aggregation with *P. loescheii* cells. At a pH of 6.8, the aggregated form of the adhesin agglutinated both sialidase-treated erythrocytes and cells of *S. oralis* 34 (76).

Capnocytophaga gingivalis Adhesin - *C. gingivalis*, a Gram negative oral gliding bacterium associated with several pathological states including an unusual neutrophil disorder and infection in immunocomprised hosts (64, 65), interacts with strains of *A. israelii* in a sialic acid-inhibitable reaction (70). In contrast to *P. loescheii*, the adhesin is intercalated into the bacterium's outer membrane. Thus, ultrasonically prepared membrane vesicles retain the ability to aggregate the partner cell, *A. israelii*. As with other Gram negative oral bacteria, the absence of sufficient genetic information mandated an immunological approach to identify the adhesin. Anti-*C. gingivalis* polyclonal antisera adsorbed with adhesin-deficient mutant cells and adhesin-specific MAbs capable of inhibiting the aggregation reaction, provided evidence that the adhesin consisted of a 150 kDa subunit polypeptide (77 - 79). Immunoelectron microscopy showed that the adhesin occurred randomly along the length of the bacterium's outer membrane.

Fusobacterium nucleatum Receptor Protein - *F. nucleatum* is a major constituent in microbial plaque deposits associated with gingivitis and periodontitis (58, 59). Strains of this Gram negative bacterium interact with *Streptococcus sanguis* producing the typical "corn cob" configurations commonly seen in plaque deposits. A hydrophobic 39.5 kDa polypeptide isolated from the cell envelope of *F. nucleatum* inhibited coaggregation when preincubated with the streptococcal cells. Antiserum prepared against the partially purified polypeptide inhibited coaggregation and agglutinated streptococcal cells coated with the cell envelope protein (80).

Gram Positive Oral Bacterial Adhesins. Fimbriae, designated as type 1 (not related to the mannose-sensitive fimbriae on *E. coli*), on *Streptococcus sanguis* strain FW 213 possess an adhesive activity that allows this bacterium to colonize the surface of a saliva coated tooth (81). Polyclonal antisera prepared against fimbriated *S. sanguis* cells and adsorbed with non-adherent mutants blocked attachment in a model system, saliva-coated hydroxyapatite (SHA) beads. Adsorbed polycolonal antiserum also bound to fimbriae and the areas were visualized with immunoelectron microscopic experiments. The adhesin appears to recognize one of the constituents in human saliva which is deposited on the tooth surface. A gene encoding the adhesion fimbriae was cloned into *E. coli* (82). Expression of a 30 kDa gene product was detected by developing immunoblots prepared from denaturing polyacrylamide gels with the adsorbed antiserum. Subsequently, the gene encoding the 30 kDa subunit was sequenced and characterized; insertional mutagenesis obliterated immunoreactivity of the polypeptide (83).

An adhesin from a related organism, *S. sanguis* strain 12, was cloned and the protein consisted of a 36 kDa polypeptide that, like the *S. sanguis* strain FW 213 adhesin, interacted with a receptor in human saliva (84, 85). Antiserum prepared against the purified gene product isolated from *E. coli* lysates inhibited attachment of *S. sanguis* strain 12 in the SHA model system. In contrast to the FW 213 adhesin, immunoelectron microscopy indicated that the antiserum prepared against the strain 12 antigen was not specific for the fimbriae on the surface of the cells. Furthermore, antiserum raised against the strain 12 fimbriae did not react with the adhesive protein. It appeared, therefore, that the strain 12 antigen was an adhesive protein which was quite different from the polypeptide identified as the adhesive fimbrial protein of strain 213. However, when the nucleotide sequence of the strain 12 adhesin was determined, it showed a high degree of

homology with the strain 213 gene (86). This intriguing contradiction has yet to be resolved, but, if the precedent set by the Gram negative bacteria can be extended to Gram positive bacteria, the protein exhibiting an adhesive function is likely to be a minor component associated with a fimbrial structure.

Attachment Versus Release - Relatively little work has been published describing how or whether bacteria, once attached to a substrate, are released. One of the few bonafide examples of a rather sophisticated release mechanism was described for a marine bacterium, *Vibrio furnessi* (87). The organism remains attached to a polysaccharide-containing substrate (glucose moieties) so long as it undergoes active metabolism or growth. Any interruption of the organism's metabolic processes, viz. nutrient depletion or addition of metabolic inhibitors, causes the immediate release of the microbe. An oral bacterium, *P. gingivalis*, appears to possess both a special binding protein which recognizes fibrinogen and a trypsin-like protease which work in concert to cleave the protein prior to its degradation (88, 89). Whether the two bacterial surface proteins constitute an attachment-release mechanism remains to be seen. Several preliminary reports have speculated that an ancillary role of some extracellular proteases produced by oral bacteria might be to alter host surfaces making attachment feasible, but neither considers the release of the bacteria under conditions of environmental stress (90, 91). A protease found in culture supernatants of *P. loescheii* is capable of degrading this organisms' 75 kDa streptococcal-specific adhesin. This protease could conceivably serve as a release mechanism under conditions of nutritional depletion. It is not yet clear whether the majority of pathogenic bacteria possess release mechanisms. Teleologically, however, it could be argued that such mechanisms would be self-defeating and that they would only serve to interrupt a highly evolved and complex infectious process.

Therapeutic Uses of Adhesins - If specific adhesins are essential for the colonization of bacteria, can they be used to interdict the disease processes caused by the accumulation of pathogens in a host? From the studies summarized in this review, the most obvious clinical uses of bacterial adhesins appear to be in the preparation of vaccines to prevent a variety of bacterial diseases. However, where topical therapies are feasible, the adhesin itself (prepared in quantity by use of the appropriate recombinant DNA technologies) or synthetically produced peptides replicating only the binding site of the adhesin might be applied directly to susceptible sites prior to or during the initial stages of infection. It is virtually predictable, from the current state of knowledge, that vaccines incorporating adhesins of the pyelonephritic strains of *E. coli* will be among the first tested to prevent recurrent bladder and kidney infections. Similarly, inclusion of the *B. pertussis* filamentous hemagglutinin as an adjunct to a genetically altered pertussis toxin would be expected to improve the efficacy of a synthetic vaccine to combat whooping cough. Little published information on the use of adhesins as antigens for vaccines is available at this time. However, the fact that a number of national and international patents proposing the use of bacterial adhesins as vaccines have already been issued reflects the optimism in such an approach to disease prevention.

Conclusions - Bacterial adhesins serve a common function irrespective of the nature of the microbe and its habitat, yet they represent a highly diverse array of proteins varying in size from 16 to 200 kDa. Few have been completely characterized structurally and little is known about their functional domains. Where genetic information is available, genes coding for adhesins appear to be components of large transcriptional units suggesting that their translocation through bacterial membrane(s) and placement at the cell surface is a complicated process. Regulation of adhesin synthesis also is not well understood and the role of environmental factors on their production is a relatively new area of investigation. Future studies are likely to address questions concerning regulation of adhesin expression in relation to expression of fimbriae, evolutionary relationships among adhesive proteins and global regulation of both fimbriae and adhesins.

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Chapter 26. Regulation of the ras GTPase Cycle

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Introduction - $p21^{ras}$ is a guanine nucleotide binding protein that is often found mutated in human tumors (1,2). In mammals, three genes encode highly homologous p21 proteins: Ki-ras, Ha-ras, and N-ras. Like other guanine nucleotide binding proteins, $p21^{ras}$ cycles between an active GTP-bound state and an inactive GDP-bound state (3,4). Oncogenic mutations of $p21^{ras}$ serve to trap the GTP-bound state (5-7). Therefore, constitutively active $p21^{ras}$ proteins lead to uncontrolled cellular proliferation. While the exact role of $p21^{ras}$ in the development of tumors is still uncertain, in adenocarcinoma of the lung oncogenic mutations correlate with poor prognosis, resistance to treatment (8), and development of metastasis (9). Although little is known about the signaling pathways involved, drugs which block the effects of $p21^{ras}$ are attractive candidates for cancer therapeutics. Here we review recent developments on the regulation of $p21^{ras}$ activity.

THE GTPASE CYCLE

The two-state model for ras action is illustrated in Figure 1. When $p21^{ras}$ is bound to guanosine 5'-diphosphate (GDP), no active signal is transmitted. Exchange of guanosine 5'-triphosphate (GTP) for GDP occurs in vivo through the action of a guanine nucleotide releasing

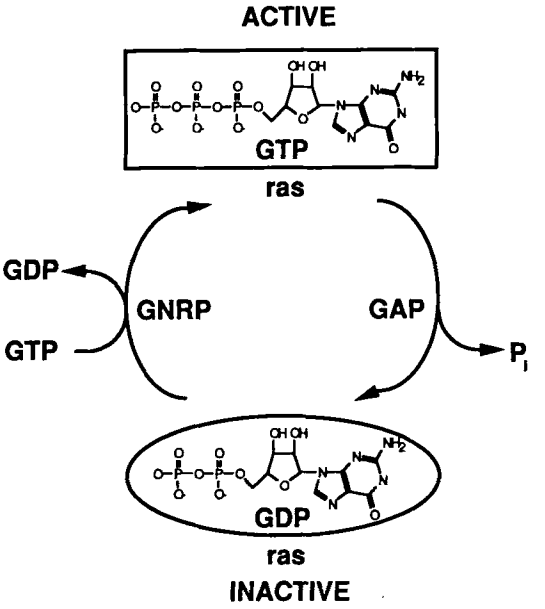


Figure 1. The ras GTPase cycle. The active GTP-bound form is converted to the inactive GDP-bound form by a specific GTPase Activating Protein (**GAP**). Release of GDP catalyzed by a Guanine Nucleotide Releasing Protein (**GNRP**) and subsequent binding of GTP reconstitutes the active form.

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protein (GNRP). The GTP-bound $p21^{ras}$ is now competent to signal, presumably by binding to an "effector" which generates a second messenger. Termination of signaling is accomplished by hydrolysis of the γ-phosphate, and this step is catalyzed by a GTPase activating protein (GAP). In order to underscore the importance of this cycle in ras action, it is instructive to examine the GDP/GTP cycle in two other systems.

Bacterial Elongation Factor Tu - In bacterial translation, the elongation factor Tu (EF Tu) functions to couple the transfer of amino acids from tRNA to the nascent polypeptide chain (10). EF Tu is a guanine nucleotide binding protein. It appears that the guanine nucleotide releasing protein (GNRP) for EF Tu is the elongation factor Ts (EF Ts). GTP-bound EF Tu binds to aminoacyl-tRNA and to the ribosome. Hydrolysis of GTP to GDP on EF Tu is catalyzed by a component of the ribosome, probably the ribosomal protein L7/L12. Concomitant with hydrolysis, the amino acyl-tRNA is transferred to the ribosome. In this example, EF Ts (the "GNRP") is apparently unregulated while the GTPase activity is regulated by codon-anticodon interactions on the ribosome (the "GAP"). It is interesting to note that certain antibiotics such as kirromycin block the action of EF Tu (11).

Heterotrimeric G proteins - A wide variety of G proteins are involved in transducing signals in eukaryotic cells (12,13). These G proteins are generally αβγ heterotrimers, with the α-subunit serving as guanine nucleotide binding protein (14). Here, the GNRPs for the α-subunit are cell surface receptors which span the membrane seven times (15). No exogenous GAP activity is known for these G protein α-subunits, and it appears that they possess a constitutively high intrinsic GTPase activity (16). On the other hand, the cell surface receptors are regulated in their GNRP activity by activating ligands. Therefore, in this example regulation of the GTPase cycle occurs at the the level of nucleotide exchange. Again it is interesting to note that certain bacterial toxins target G protein α-subunits (17).

In these two examples, the GTPase cycle is regulated at different steps. As described below, it appears that mammalian $p21^{ras}$ is regulated through its GAPs in a similar manner as EF Tu.

THE ras GTPASE CYCLE

Guanine nucleotides bind very tightly to the $p21^{ras}$ protein. While both GDP and GTP bind very fast (on rate essentially diffusion limited), both nucleotides are released very slowly (off rate ~ 0.05 min^{-1}) (18-20). In addition, the $p21^{ras}$ protein possesses a very slow intrinsic GTPase activity (~ 0.02 min^{-1}) (19-22). Since exchange of GTP for GDP and hydrolysis of GTP are very slow, $p21^{ras}$ requires both GNRPs and GAPs in order to cycle efficiently.

Oncogenic mutations in $p21^{ras}$ are found in regions of the protein which are known from X-ray crystallography to interact with the guanine nucleotide (23-27). All mutations that are found in tumors decrease the intrinsic GTPase activity. It has been shown for the most common mutations (position 12 and 61) that almost every amino acid substitution results in reduced GTPase activity (28,29). From these early data, it was reasoned that oncogenesis was promoted by a block to the GTPase cycle. Confirmation of this idea has come from an examination of the GNRPs and GAPs of $p21^{ras}$.

For $p21^{ras}$, the GNRPs are just becoming characterized, while GAP activity has been studied for several years. In this section, we will briefly review the recent work on GNRPs and highlight the GAPs. In addition, we will discuss the yeast system recently reviewed in (30,31), where RAS function has been amenable to genetic characterization.

ras Guanine Nucleotide Releasing Proteins - ras GNRP activities have been localized to both cytosolic (32,33) and membrane fractions (34,35). Several main points should be highlighted. The GNRPs are much less selective than the GAPs, since several small guanine nucleotide binding proteins other than ras are substrates (35). Oncogenic mutants of $p21^{ras}$ appear to be utilized as substrates (33).

In the yeast *Saccharomyces cerevisiae*, two GNRPs for the yeast RAS genes have been described (36-38). These proteins, CDC25 and SDC25, were originally identified through genetics: mutants in CDC25 are defective in the RAS pathway and the carboxyl-terminal domain of SDC25 is able to complement a CDC25 mutation. Recently, it has been shown biochemically that the carboxyl-terminal domain of SDC25 does indeed function as GNRP for both yeast RAS and

human p21^{ras} (38). However, unlike CDC25, SDC25 is not an essential gene, suggesting it may be a GNRP for a different pathway (39). As will be described below, one effector of RAS in yeast is known to be adenylate cyclase. Although it appears that adenylate cyclase is not a mammalian p21^{ras} effector (40,41), the amenability to genetic dissection make the yeast system an attractive model to analyze the ras GTPase cycle as a biological switch.

ras GTPASE ACTIVATING PROTEINS

Three ras-specific GAPs have been found in mammalian cells and two GAPs have been found in the yeast *Saccharomyces cerevisiae*. The mammalian proteins include two forms of the originally identified protein, specified p120- and p100-GAP according to their molecular weights, and an additional protein encoded by the neurofibromatosis type 1 gene, referred to as NF1-GAP (42-44). In yeast, the GAPs are encoded by the genes IRA1 and IRA2 (45,46). In Figure 2, a schematic representation indicates the regions of homology. It is clear from this figure that only a small portion of each protein is dedicated to GTPase activation. NF1-GAP shows homology to the IRA proteins over a considerably larger region than the domain thought to stimulate the GTPase of p21^{ras}. Unlike NF1-GAP and the IRA proteins, p120-GAP also shares homology with noncatalytic regions of the src oncogene (src homology regions 2 and 3, SH2 and SH3) (47). These observations indicate that these proteins may serve other functions in addition to terminating the ras signal. We will review evidence which suggests that the GAPs may actively propagate the signal.

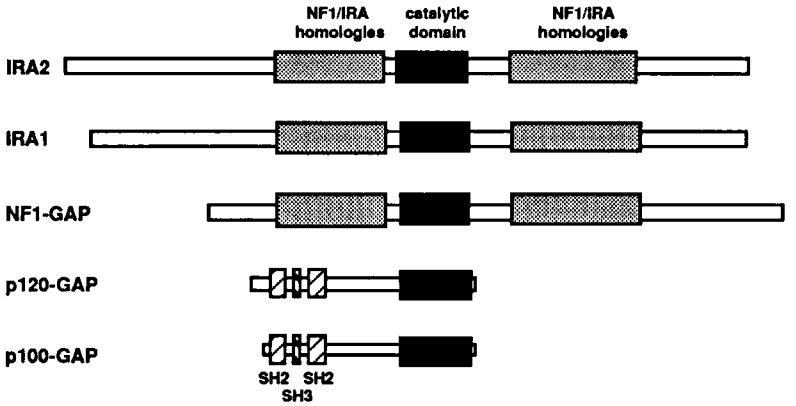


Figure 2. Representation of sequence homologies among different GAPs.

p120- and p100-GTPase Activating Proteins - GAP activity was initially identified in cytosolic extracts of *Xenopus laevis* oocytes (48). Subsequently, this activity was purified from bovine brain (49) and cloned from bovine brain (43) and human placenta (42). The GAP proteins from both bovine brain and human placenta shared over 96% amino acid identity. cDNA clones from both tissues were found to encode proteins of about 120 kDa (p120-GAP). In human placenta, a second clone resulting from alternative mRNA splicing was also found which encoded a truncated protein of 100 kDa (p100-GAP), missing a hydrophobic N-terminal region (42). This second form has so far been found only in human placenta (50). No clear biochemical differences between these GAPs have been established. It has also been shown that a fragment of p120-GAP comprising only the carboxyl-terminal third of the protein is competent to catalyze hydrolysis of GTP on p21^{ras} (51).

p120-GAP catalyzes the hydrolysis of GTP to GDP on wildtype but not oncogenic mutants of p21^{ras} (48). Therefore, it has been proposed that the accumulation of GTP-bound oncogenic p21^{ras} in transformed cells is due to the inability of GAP to catalyze GTP hydrolysis. Interestingly, binding of GAP to these mutants is not impaired (43). This information has contributed to the idea that GAP may play a second important role in p21^{ras} signaling, acting as an effector to transmit a signal.

Other data support the hypothesis that GAP may be an effector of ras action. Although the effector in the p21^{ras} signaling pathway has not been identified in mammalian cells, one region of p21^{ras} has been implicated as the site where the effector binds. Mutations in this region (amino acids 32 to 40) lead to p21^{ras} proteins that are defective in signaling (52). These mutants are poor

substrates for GAP and it has been concluded that GAP binds to this "effector-binding" site. Indeed, these mutants bind weakly to GAP (43).

More evidence to support this hypothesis has come from experiments designed to find inhibitors of ras action. Proteins which cause reversion of transformation by an oncogenic K-ras gene were selected (53). One of these proteins was isolated and named Krev-1 (also known as rap1 and smg p21). This protein is a small guanine nucleotide binding protein which is closely related to $p21^{ras}$ (~ 50% homology); indeed Krev-1 and $p21^{ras}$ are identical in the effector binding site. Furthermore, Krev-1 binds to p120-GAP with a much higher affinity than $p21^{ras}$ (54,55). Therefore, it has been suggested that Krev-1 blocks $p21^{ras}$ transformation by binding to and sequestering p120-GAP. If the only role of p120-GAP is to catalyze hydrolysis of GTP on $p21^{ras}$, the result of sequestering p120-GAP would be an accumulation of $p21^{ras}$•GTP and hence increased transformation. Since Krev-1 has the opposite effect, namely reversion of transformation, perhaps the primary consequence of Krev-1 expression is to block the effector function of p120-GAP.

Recent analysis of the $p21^{ras}$ effector site has provided further support for the hypothesis that p120-GAP is an effector. Effector site mutations do not block transformation if a second, activating, mutation exists on the protein (56). The authors conclude that, while the double mutant binds weakly to p120-GAP, the primary consequence is a pronounced accumulation of $p21^{ras}$ in its GTP-bound state. This accumulation overcomes the lowered affinity for p120-GAP, so the signaling $p21^{ras}$•GTP / p120-GAP complex still forms.

Direct evidence for a p120-GAP signaling role has recently been reported. In guinea pig atrial membranes, p120-GAP and $p21^{ras}$ collaborate to block the G protein-activated opening of potassium channels (57). p120-GAP is absolutely required in addition to $p21^{ras}$, and antibodies to either p120-GAP or $p21^{ras}$ specifically inhibit the effect. From these results, it was speculated that the $p21^{ras}$ / p120-GAP complex actively generates a signal which blocks opening of the channel.

It should be noted that a study in which p120-GAP overexpression was shown to revert transformation in fibroblasts argued against the idea that p120-GAP was the only effector (58). However, the discovery of at least one additional GAP (NF1-GAP, see below) reconciles this result with the notion of GAPs as effectors.

p120-GAP is a substrate for activated PDGF receptors (59), thus establishing a link between p120-GAP and tyrosine kinase signaling pathways. Indeed, p120-GAP binds extremely tightly to activated PDGF receptors (60). In addition to p120-GAP, phospholipase C-γ, phosphatidylinositol 3-kinase and the raf oncogene (a serine/threonine kinase) are also associated in this complex (61). Other growth factor receptors and the src tyrosine kinase also phosphorylate and associate with p120-GAP (62). In addition, p120-GAP binds extraordinarily tightly to two tyrosine-phosphorylated proteins of molecular weights 62 and 190 kDa; little else is known about these proteins. It appears that the interaction of p120-GAP and the phosphotyrosine containing proteins occurs via the SH2 domains of GAP (63,64). Although the functions of these complexes is still unknown, it is interesting to note that $p21^{ras}$ is implicated in the signaling pathways of many of these growth factors and oncogenes (65,66).

A link with other signaling pathways is suggested by the regulation of GAP activity by second messenger lipids. It has been shown that arachidonate, phosphatidate, and inositol phospholipids inhibit GAP activity in extracts (67-69). A provocative speculation may be that these lipids prolong the activity of the $p21^{ras}$ / GAP complex. More recently, lipid activators of GAP activity has been described (70): prostaglandins $F_{2\alpha}$ (9-α) and A_2. This activation is very specific, since prostaglandins $F_{1\alpha}$ and an isomeric $F_{2\alpha}$ (9-β) do not activate. Interestingly, this activation is competitive with arachidonic acid, the precursor of the prostaglandins. Therefore, it is conceivable that arachidonate and the activating prostaglandins regulate p120-GAP by competing for the same site as depicted in Figure 5.

NF1-GTPase Activating Protein - Von Recklinghausen Neurofibromatosis Type I is an autosomal dominant genetic disease, mainly characterized by a certain type of skin pigmentation, the so called cafe-aut-lait-spots (CLS), and benign neurofibromas (71). This disease, affecting around 1 in 3500 individuals in all ethnic groups, is progressive, in some cases eventually leading to severe disfiguration and appearance of malignant tumors, e.g. neurofibrosarcomas. Other less common features are Schwannomas, optic gliomas and learning disabilities.

The NF I gene was mapped to chromosome 17 (17q11.2; 72,73) and subsequently the 3' part of the cDNA was sequenced (44,74,75). The mRNA sizes were estimated to be around 13 kb by Northern blot analysis. The predicted partial protein sequence showed homologies to the yeast IRA 1 and 2 proteins and the mammalian GAP gene product. A more detailed sequence comparison revealed that the homology to p120-GAP was restricted to the catalytic domain, whereas homology to IRA 1 and 2 was significantly extended to either side of this domain.

The part of the NFI gene related to the catalytic domain of p120-GAP/IRA stimulates the GTPase activity of $p21^{ras}$ (76-78). This GTPase stimulation by NF1-GAP is specific for ras; the related proteins rap 1 and Sec4 are unaffected. Also, NF1-GAP is incapable of stimulating the GTPase activity of oncogenic mutants of $p21^{ras}$. A biochemical comparison revealed that NF1-GAP binds 20-times more tightly to $p21^{ras}$ ($K_d \sim 250$ nM compared to 5 μM), but has a specific activity around 30-fold lower than p120-GAP (20 pmol min^{-1} compared to 660 pmol min^{-1}), i.e. at low concentrations of $p21^{ras}$ the stimulation of hydrolysis is comparable for both proteins, whereas at high concentration p120-GAP activity dominates (76).

In yeast NF1-GAP, like p120-GAP, can complement the increased heat shock sensitivity of IRA 1 and 2 mutations, and it also inhibits the effects of human ras expressed in yeast. NF1-GAP, in contrast to p120-GAP, also inhibits effects due to expression of an activated H-ras gene, and this is probably due to the higher affinity of NF1-GAP for $p21^{ras}$ (76-78).

Whether the kinetic differences have a biological function is unclear, because the amounts of p120-GAP/NF1-GAP and active RAS in different cell types are not yet known. Nonetheless, Northern blot analysis has revealed that the NF I gene, like p120-GAP, is ubiquitously expressed and Southern blots have shown evolutionary conservation among a wide variety of species (79).

So far little is known about the function of NF1-GAP, besides downregulation of $p21^{ras}$ proteins. In NF I patients, the NF1 gene is often found mutated outside of the GAP catalytic domain. It is worth noting that in some tissues affected in NF I patients (e.g. glial cells, neurons and phaeochromocytoma cells) ras is known to induce differentiation (80-82).

GAP regulation of ras - Several systems have been described recently in which $p21^{ras}$ activation has been directly linked to regulation of GAP activity. In antigen-stimulated T cells, $p21^{ras}$ accumulates in its GTP-bound state (83). This effect proceeds via a protein kinase C mediated pathway, and the key factor appears to be inhibition of GAP activity. In fibroblasts, a similar result is obtained by insulin activation, except it appears that here protein kinase C is not involved (84). In both systems, it seems that regulation of GAP activity may be critical. In fibroblasts, $p21^{ras}$ also accumulates in the GTP-bound state in response to other growth factors and related oncogenes (85-87) although the connection with GAP has not been established.

Yeast GTPase Activating Proteins - While CDC25 was identified as an activator of RAS in *Saccharomyces cerevisiae*, the IRA1 and IRA2 proteins have been genetically defined as inhibitors of RAS action (45,46). Sequencing of the genes revealed a region of homology with p120-GAP and more extensive homology with NF1-GAP (Figure 2). Although extracts derived from yeast cells display no GAP activity on mammalian $p21^{ras}$, it appears that mammalian GAPs are active on the yeast RAS genes. Thus, p120-GAP (88,89) and NF1-GAP (76-78) suppress IRA mutations. More recently, a portion of the IRA2 gene has been shown to possess GAP activity in vitro (90). Therefore, the critical components of the GTPase cycle in *Saccharomyces cerevisiae* have been identified as depicted in the model of Figure 3 (for recent reviews see 30,31) Here, the GTPase cycle is regulated by the GNRP CDC25, and by the GAPs IRA1 and IRA2. RAS.GTP activates adenylate cyclase (CYR1) either directly or via interaction with a subunit called CAP (for Cyclase Associated Protein) or SRV2 (91,92). Nutrient limitation induces cyclic AMP accumulation via the RAS pathway. However, it is unclear if this is done by activating the GNRPs or by inhibiting the GAPs. Although the yeast system contrasts sharply with the mammalian one in terms of effector function, it will be interesting to compare how the GTPase cycles are regulated.

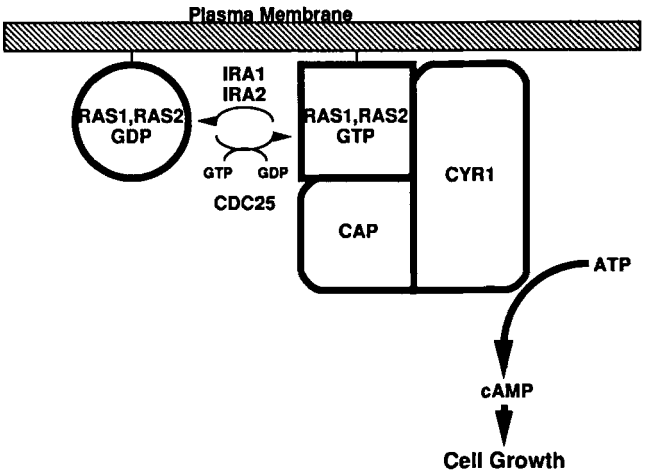


Figure 3. Regulation of the RAS GTPase cycle in Saccharomyces cerevisiae. The interconversion of the GDP- and GTP-bound forms of the yeast RAS proteins (RAS1 and RAS2) is achieved through two GAPs, IRA1 and IRA2, and through at least one GNRP, CDC25. Activated GTP-bound RAS stimulates the adenylate cyclase activity of CYR1, an event mediated by CAP protein.

GTPASE ACTIVATING PROTEINS FOR ras-RELATED PROTEINS

rap1-GAP - For rap1, three different specific GAPs have been described: two cytosolic forms in bovine brain (93) and human platelets (94), and an 88 kDa membrane associated protein in HL60 cells (95). Although rap and ras proteins share identical effector regions, none of these rap1-GAPs are able to stimulate hydrolysis of GTP bound to ras. Since GTP-bound rap1 inhibits the $p21^{ras}$-specific p120-GAP, rap1-GAP may be a useful drug target. By inhibiting rap1-GAP activity, a drug could raise the level of rap1 in its GTP-bound form and consequently sequester p120-GAP.

Other GTPase Activating Proteins - The high specificity of ras- and rap-GAPs suggests that every other small GTP-binding protein has its own GAP. So far GAP activities have been described for all three main subgroups of ras-related proteins.

For rho, a protein apparently involved in organizing the cytoskeletal network (96), a 29 kDa rho-GAP has been characterized (97). GAP activities have been described for the large group of vesicle targeting ypt/rab proteins including ypt1/rab1 (98) and rab3A (99). To date, none of these proteins have been cloned or sequenced, but it will be very interesting to see whether these proteins are related, like their substrates, or completely divergent.

POSTTRANSLATIONAL MODIFICATION OF ras PROTEINS

In order for $p21^{ras}$ proteins to effect biological activities, they must be localized to the membrane. A cascade of posttranslational modification, commencing with farnesylation of cysteine residue 186 (100-102), is required for membrane localization of mammalian $p21^{ras}$ (Figure 4). Cysteine 186 is followed by two generally aliphatic amino acids (A) and a third residue (X) not conserved among the ras superfamily of proteins: these residues form the CAAX box that is processed as illustrated in Figure 4. In the yeast *Saccharomyces cerevisiae*, the farnesyltransferase is apparently encoded by two genes which may act as separate subunits (103,104). The mammalian farnesyltransferase has been characterized (105-108), and drugs which inactivate this enzyme are being sought. Farnesylated ras is further processed by proteolysis and methylation (101,109,110), as indicated in Figure 4. These modifications are essential for efficient membrane binding (111). Although further modification is not absolutely required for membrane localization, palmitoylation enhances the activity of some of the ras proteins (112,113). Inhibitors of the reactions illustrated in Figure 4 should block the membrane association and hence the biological activity of the ras proteins. Indeed, cytosolic forms of activated ras proteins interfere with the function of the

endogenous membrane-bound ras proteins (30,114). Therefore, drugs which target ras processing enzymes may selectively impair proliferation of ras-transformed cells (115,116).

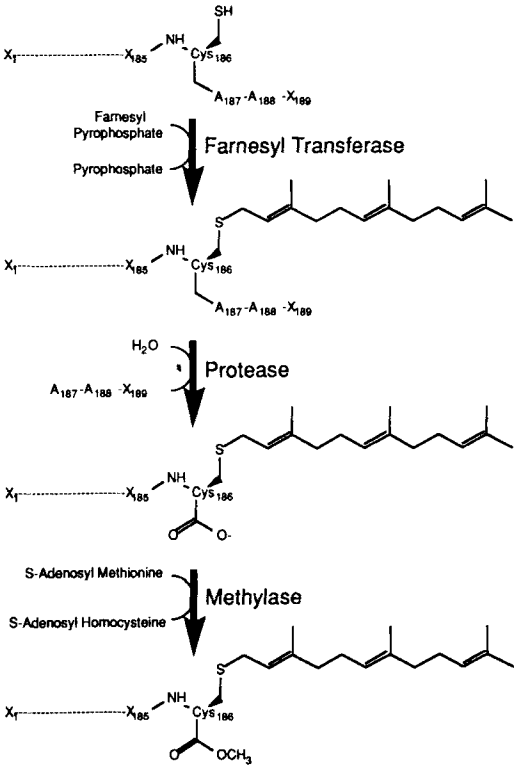


Figure 4. Posttranslational Modifications of ras Proteins. Farnesylation at cysteine residue 186 of human ras proteins is followed by removal of the three carboxy-terminal amino acids and methylation of the new terminal carboxylate. The residues surrounding cysteine-186 are indicated as A (aliphatic) or X (unconserved) depending on homologies among the ras proteins.

PROSPECTS FOR THERAPEUTICS

Since $p21^{ras}$ plays a central role in proliferation and differentiation, it is crucial that drugs target activated pathways specifically. In Figure 5 a putative mammalian $p21^{ras}$ GTPase cycle is illustrated, highlighting the areas where drug targeting may inhibit tumor progression. As described above, GNRPs regulate binding of GTP to $p21^{ras}$, while GAPs regulate GTP hydrolysis. We have reviewed the recent evidence that suggests that the GAPs are putative targets of $p21^{ras}$ and that GAP regulation may be critical in controlling $p21^{ras}$ activation. If the model is correct, four major drug targets are apparent: (A) the GAP effector activities, (B) GTPase regulation, (C) $p21^{ras}$ directly and (D) posttranslational modification. (A) Targeting the effector activities directly, while imparting the most selectivity, must probably await their direct biochemical identification. However, molecular cloning of both p120-GAP and NF1-GAP affords an opportunity to reintroduce mutated proteins into biological systems, thereby identifying the most attractive protein sequence targets. Indeed, a catalog of the naturally occuring NF1-GAP mutations which cause neurofibromatosis should provide an initial map of the NF1 protein. (B) Agents that affect the GTPase activity of oncogenic $p21^{ras}$ proteins could either act on endogenous GAPs or replace them. It is conceivable, since both p120-GAP and NF1-GAP still bind to oncogenic mutants, that drugs could modify GAP activity so as to enable GTPase activation. Rational design of such drugs would depend on a thorough understanding of the mechanism of GTPase activation. Such an understanding may also facilitate the design of agents which could independently downregulate the oncogenic proteins. (C) The

availability of a three-dimensional structure for wild type and oncogenic $p21^{ras}$ proteins may hasten design of direct selective inactivators. Blocking the interaction of $P21^{ras}$ with its GNRP's may also prove therapeutically useful. (D) Recent insights into posttranslational modification of the $p21^{ras}$ proteins have spawned interest in targeting the modifying enzymes. However, given the importance of ras proteins to general cellular metabolism, the most selective approaches, such as aiming at specific ras effectors, may prove to be the most successful.

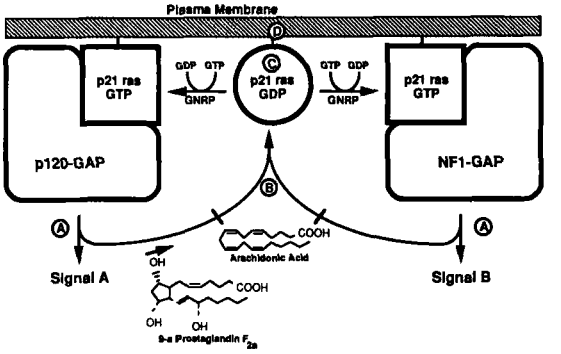


Figure 5. Schematic depiction of possible therapeutic targets in the human ras pathway. Activated GTP-bound ras proteins putatively activate effector functions of the GAPs p120-GAP and NF1-GAP. Interacting drugs may affect (A) the GAP effector activities; (B) GTPase regulation; (C) p21 ras directly; or (D) posttranslational modification. As one known example, arachidonic acid blocks both GAP activities, while prostaglandins activate p120-GAP.

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SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

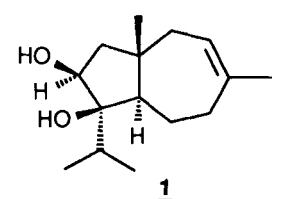
Editor: Michael C. Venuti
Genentech, Inc.
South San Francisco, California 94080

Chapter 27. The Search for Molecular Diversity (I): Natural Products

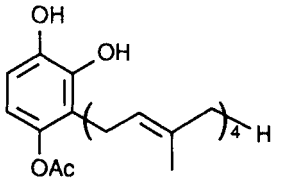
Peter J. Hylands and Louis J. Nisbet,
Xenova Limited, Slough SL1 4EF, Berkshire, England

Introduction - The search for novel pharmacophores from natural sources has continued apace over the past few years. The emphasis has changed from random screening to a more direct approach. Advances in molecular biology have allowed the development of mechanism-based receptor screens to probe interactions between large molecules and find small natural product molecules as potential agonists and antagonists. The choice of organisms to be examined has also been refined in response to the recognition that evolutionary pressures have exerted a marked effect on secondary metabolite production. This is particularly true for those organisms living in close association with each other. Special emphasis has thus been recently placed on the selection of those types of organisms as the raw material for screening programmes. This chapter provides a selective, illustrative account of the literature since one of us reported on this subject (1), but with special emphasis on data published in 1990. Material included is chosen to highlight chemical diversity and is divided into a number of sections based on the origin of the producing organisms: fungi, actinomycetales, marine organisms (including algae), and plants.

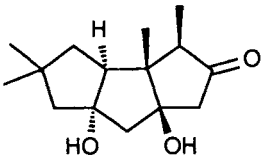
Fungi - A novel carotane-type sesquiterpene, CAF-603 (1) has been isolated from *Gliocladium virens* IFO 9166 (2); it is active against yeasts and dermatophytes.



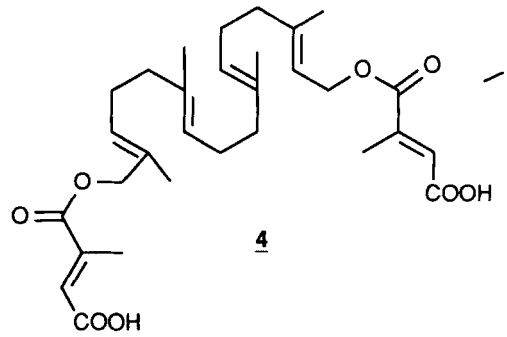
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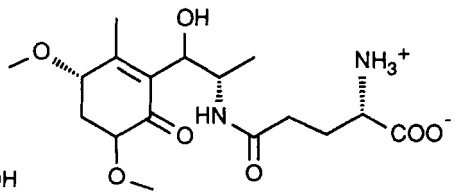
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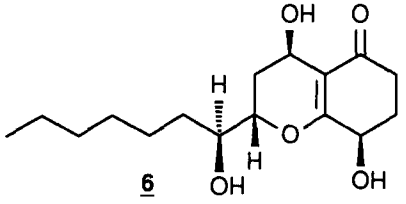


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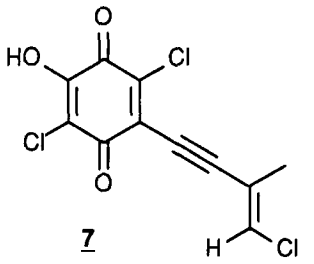
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Problems with isolation, culture and preservation of the higher fungi are gradually being overcome: basidiomycetes are thus being more extensively studied. Fruit bodies of the basidiomycete *Suillus granulatus* have yielded, in addition to suillin (2), a series of related phenols with antimicrobial activity (3). An unidentified basidiomycete has yielded arthrosporone (3) and other potent antifungal sesquiterpenoids (4). The cavipetins (such as cavipetin A, 4), unusual unsaturated antifungal diterpene esters, have been isolated from *Boletinus cavipes* (5). Another basidiomycete, *Tricholoma lascivum*, has yielded lascivol (5), a bitter principle (6).

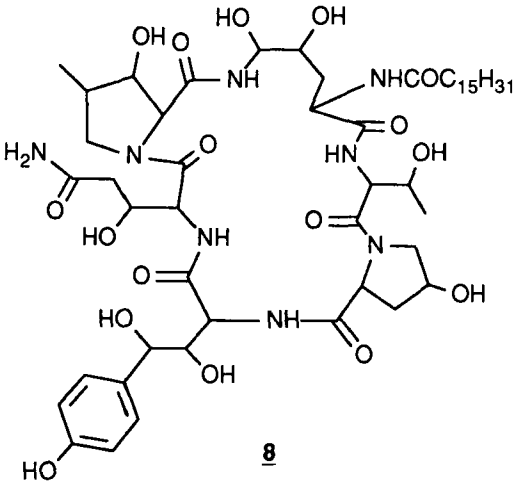
Trichoderma koningii extracts have provided a material (6), suppressive to the saprophytic growth of the take-all fungus, *Gaeumannomyces graminis* var. *tritici* (7). A new metabolite, mycenon (7), from a *Mycena* species, TA 87202, is an inhibitor of isocitrate lyase (8).



6

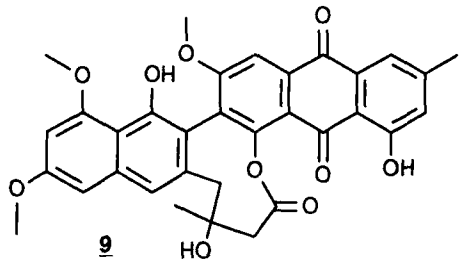


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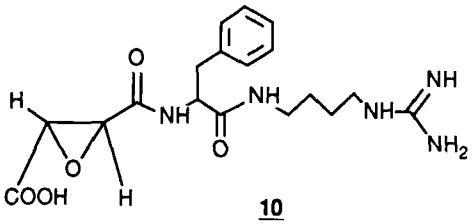


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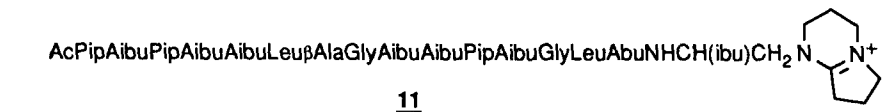
An antifungal lipopeptide, L-671,329 (8), has been isolated from the fungus *Zalerion arboricola* (9). 2-Methoxy-5-methyl-1,4-benzoquinone has been claimed to be an inhibitor of platelet aggregation (10). Unique macrocylic lactones dermocanarins I (9) and II from *Dermocybe canaria* has been isolated (11). Estratins A (10) and B are new thiol protease inhibitors from *Myceliophthora thermophila* M4323 (12).



9



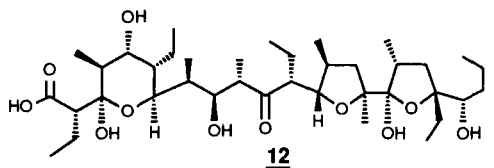
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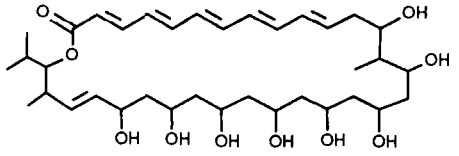
11

Entomopathogenic fungi have been the subject of continued interest: the structures of efrapeptins D (11) and F, insect toxins and potent inhibitors of mitochondrial ATP-ase from *Tolypocladium nireum* have been determined by X-ray analysis (13).

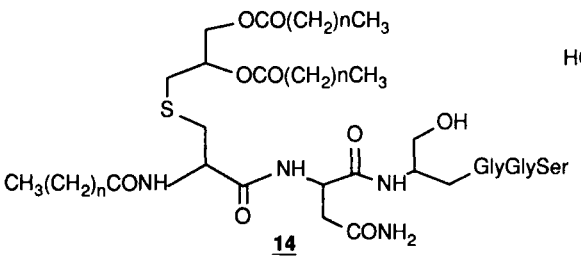
Actinomycetes - A novel polyether, inostamycin (**12**), has been isolated from *Streptomyces* sp M4816-AF15, potent at inhibiting phosphatidylinositol turnover (IC_{50} = 500 ng/mL) in cultured A431 cells (14). A new broad-spectrum antibiotic, faerlefungin (**13**), has been isolated from *Streptomyces griseus* var. *autotrophicus* (15). A new lipopeptide, W51279 (**14**), has been isolated from *Streptomyces willmorei* (16). *Streptomyces graminofaciens* BA14348 has yielded new flavonones (e.g. BE-14348A, **15**) which selectively inhibit binding of estrogens to their receptors (17). Also, WS-7528, an isoflavone (**16**) isolated from *Streptomyces* sp N.7528 inhibited estrogen binding (for the partially purified rat uterine cytosol receptors) with an IC_{50} of 5.7 x 10^{-8} M (18).



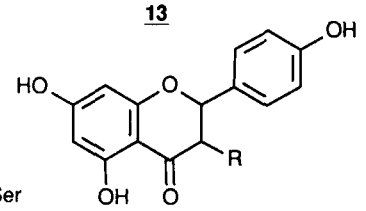
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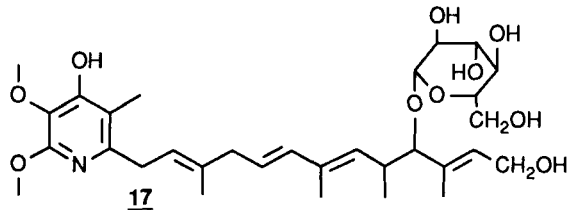


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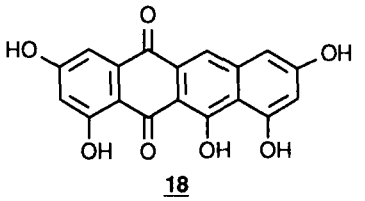


15 R=H
16 R=CH_3

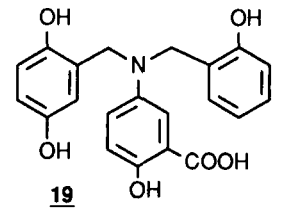
New members of the piericidin class such as 13-hydroxyglucopiericidin A (**17**) from *Streptomyces* spp SN-198 and OM-5689 have been isolated (19,20). Saintopin (**18**), a new anthraquinone, with topoisomerase II-dependent DNA cleavage activity has been isolated from a *Paecilomyces* species (21). A renin-inhibitory peptide has been isolated from a *Streptomyces* sp NR 0516 (22). A potent tyrosine kinase inhibitor, lavendustin A (**19**) has been isolated from the culture filtrate of *Streptomyces griseolavendus* (23). It inhibits epidermal growth factor receptor-associated tyrosine kinase with an IC_{50} of 4 ng/mL and does not inhibit protein kinase A or C.



17



18



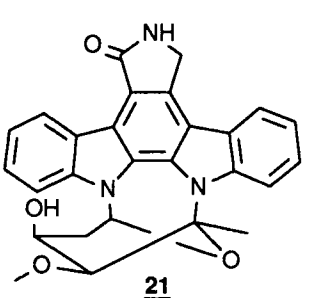
19

GlyPheIleGlyTrpGlyAsnAspIlePheGlyHisTyrSerGlyAspPhe
β

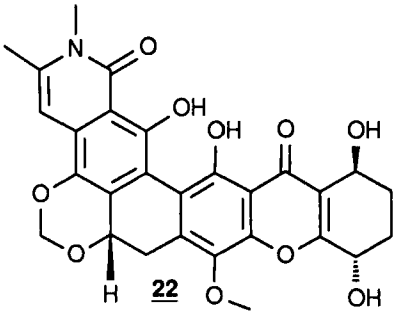
20

Anantin (**20**), a peptide binding to the receptor of the atrial natriuretic factor consists of 17 *L*-amino acids forming a cyclic system (24,25). Anantin is the first microbially-produced antagonist of the cardiac hormone, ANF, binding with a K_d of about 600 nM.

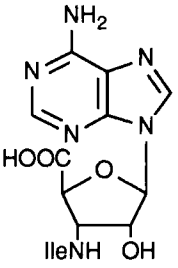
The conformation of staurosporine, a potent protein kinase C inhibitor has been determined in solution (26). A new analogue of staurosporine, RK-286C (**21**), has been isolated from a *Streptomyces* (27). A new antibacterial agent, simaomicin α (**22**), has been isolated from *Actinomadura madurae* LL-D42607 and is the most potent naturally occurring anticoccidial agent (28).



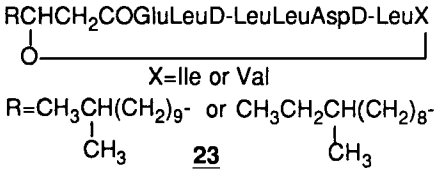
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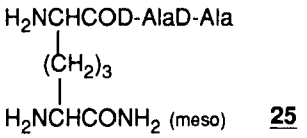
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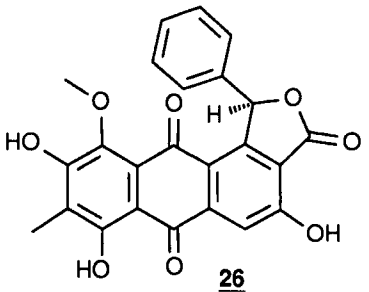
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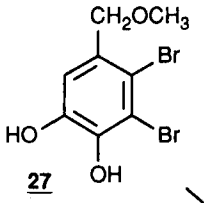


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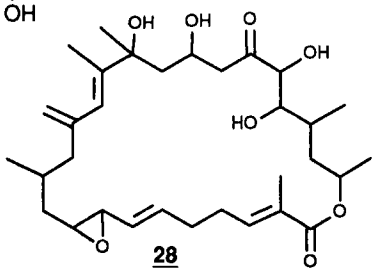


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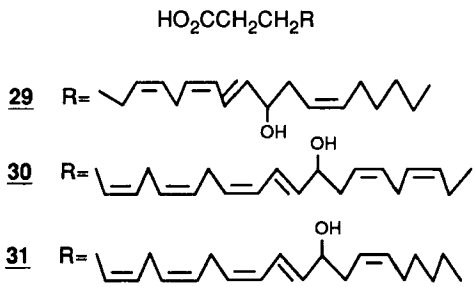
The pumilicidins such as pumilicidin A (**23**) are novel cyclic acylheptapeptides comprising a β-hydroxy fatty acid, two *L*-leucine, two *D*- leucine and *L*-glutamic, *L*-aspartic and *L*-isoleucine (or *L*-valine) residues. They inhibit herpes simplex virus type I and H^+-ATPase (29). FR-900403 (**24**) is a new antifungal antibiotic from a *Kernia* species (30). Aladapcin (**25**) is a new peptidyl microbial metabolite that enhances host resistance against bacterial infection (31). Basidifferquinone (**26**) isolated from a *Streptomyces* is a novel fruit body-inducing agent for the basidiomycete *Favolus arcularis* (32).



27



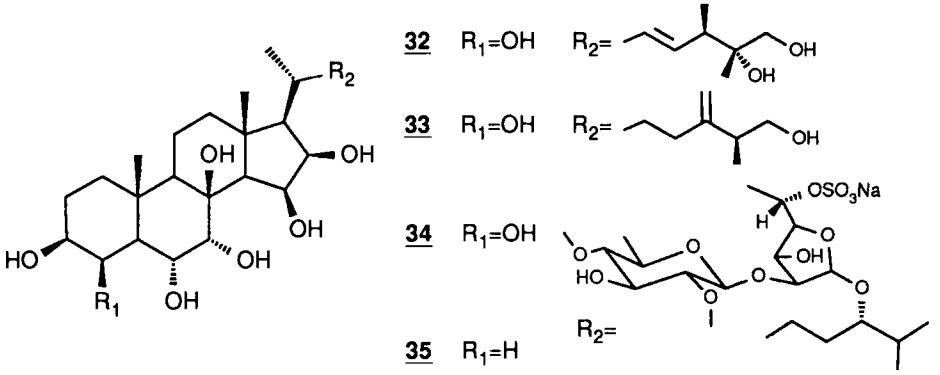
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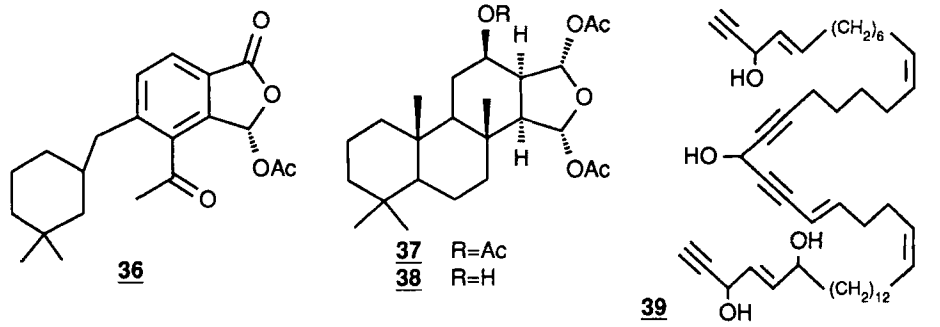
29 **30** **31**

Marine organisms - A growth stimulating-halocatechol, α-*O*-methyllanusol (**27**), from the red algae *Odonthalia washingtoniensis* and *O. floccosa*, was detected using bioassays with lettuce and rice plants (33). Extensive chemical investigations on nineteen Australian algae from the Rhodophyta, Phaeophyta, Cyanophyta and Chlorophyta have been reported (34) encompassing a total of 67 secondary metabolites of diverse biosynthetic origin. A further cytotoxic macrolide (**28**) has been isolated from a cultured marine dinoflagellate (genus *Amphidinium*) which had been in symbiotic association with a flatworm from Okinawa (35). Active metabolites from microalgae have been reviewed (36).

Large amounts of (+)-(12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydroxy-5,8,10,14-icosatetraenoic (**29**) and (+)-(12*R*,4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*)-14-hydroxy-4,7,10,12,16,19-docosahexaenoic (**30**) and (+)-(12*R*,4*Z*,7*Z*,10*Z*,12*E*,16*Z*)-14-hydroxy-4,7,10,12,16-docosapentaenoic acids (**31**) have been isolated from the demosponge *E. chinochalina mollis* (37), the first reported occurrence from a marine invertebrate.

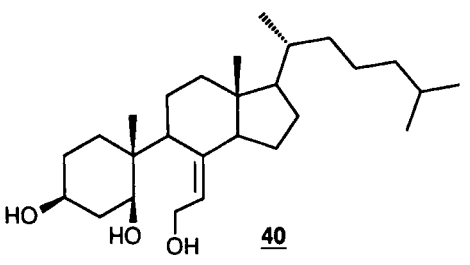


Further members (**32** and **33**) of the widely-occurring starfish steroidal sapogenin class have been isolated by the Naples group (38) from the starfish *Patria miniata*. Examples of new sulphated steroidal fucofuranosides are **34** and **35**, isolated from another starfish *Dermasterias imbricata* (39). Interesting ichthytoxic activity is shown by some new diterpenes (**36**-**38**) from the nudibranch mollusk *Chromodoris luteorosea*; it is suggested that these may play a natural role as defense allomones (40).

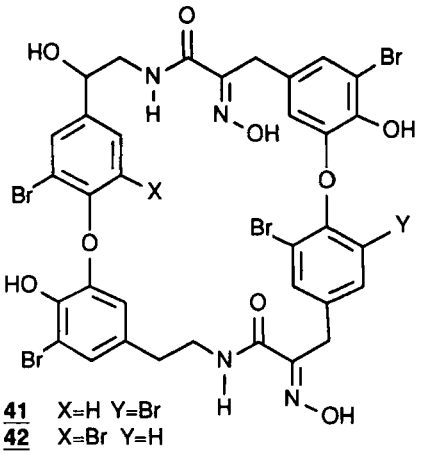


More bioactive polyacetylenes (such as **39**) have been isolated from the red sponge *Petrosia ficiformis* (41). A new type of seco-sterols has been isolated from a sponge (42). Previously the only marine sterols of this type had been 9,11-*seco*-compounds but substances of the type **40**, unusually cleaved between carbons 5 and 6 have shown to present in *Hippospongia communis* (42). The complete iso-anteiso 29:0 series of fatty acids, *i.e.*, the novel 27-methyloctasanoic and 26-methyloctasanoic acids, have been isolated from the sponge *Petrosia pellasarca*, adding to speculation on the role and complexity of fatty acid biosynthesis in marine organisms (43). The

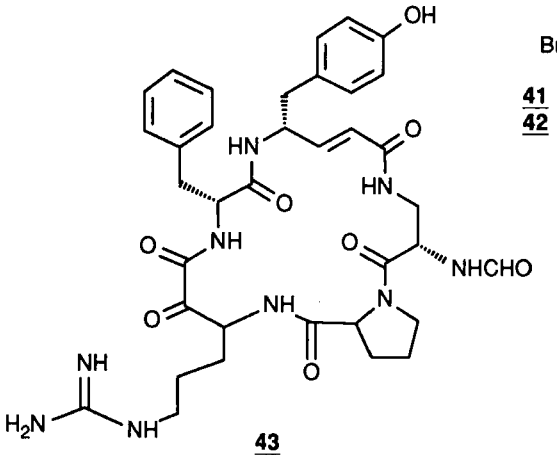
bastadin series has been extended by the discovery of bastadins 8 and 9 (**41** and **42**) from the marine sponge *Ianthella basta* (44). Powerful thrombin inhibitory activity is displayed by the cyclotheonamides, such as cyclotheonamide A (**43**) isolated from a marine sponge of the genus *Thesnella* (45).



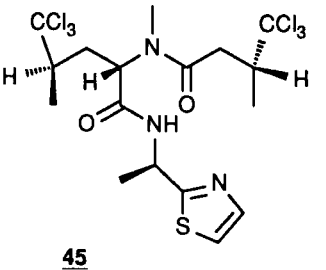
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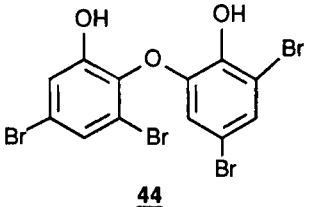
41 X=H Y=Br
42 X=Br Y=H



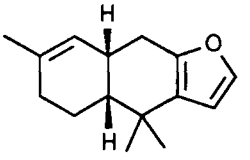
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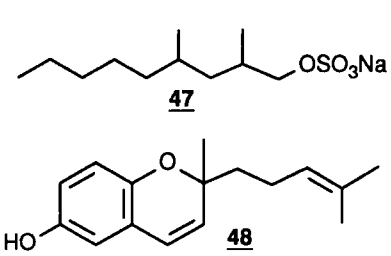
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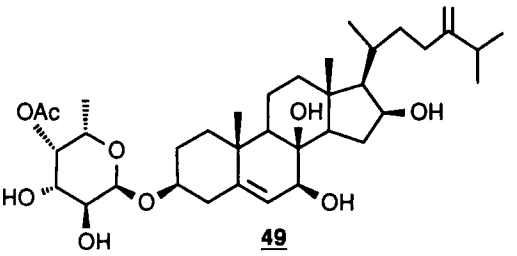


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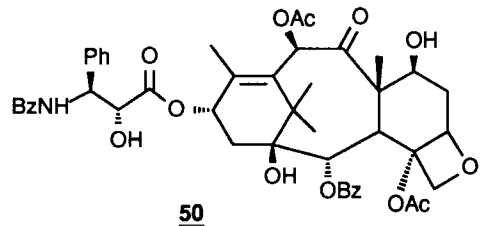
The diversity of secondary metabolism is well illustrated by work in the sponge *Dysidea*; aromatic compounds such as **44**, polychlorinated tetrapeptides, e.g., (-)-dysidenin (**45**) and polycyclic furanosesquiterpenoids such as (-)-furodysinin (**46**) have all been found in *D. heraceae* (46). The marine worm, *Polyphysia crassa*, has yielded 2,3,4-tribromopyrrole, a compound principally responsible for the antibacterial activity of the extracts (47).

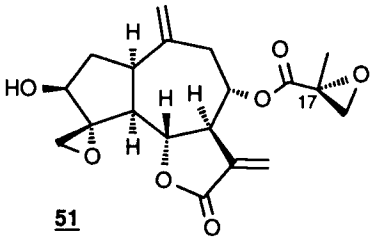
Novel sulphated hydrocarbons, e.g. **47**, have been isolated from the sea cucumber, *Cucumaria frondosa* (48). Antioxidant chromenes (e.g., **48**) have been isolated from the colonial tunicate *Amaroucium multiplicatum* (49) as a result of an antioxidant activity-directed analysis. The soft coral *Sinularia crispa* has yielded a steroid glycoside (**49**) which shows potent spermatostatic activity on rat cauda epididymmal spermatozoa (50). Reviews on marine natural products have appeared (51-53).

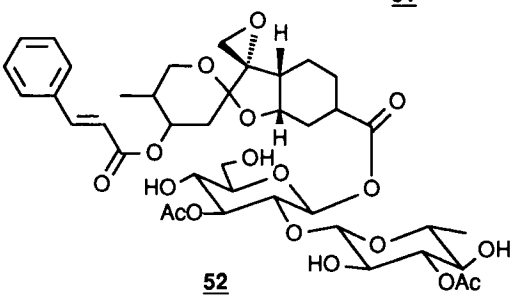




Plants - Plant products as sources of potential agents in the prevention and treatment of cancer have been reviewed (54). The chemistry of the most promising plant-derived lead compound in cancer chemotherapy, particularly in ovarian caner (55), taxol (**50**) has also been reviewed (56). Because supplies of taxol for clinical evaluation are limited, new sources of have been examined: needles from a number of *Taxus* species have been proposed as a renewable source of taxol instead of the *Taxus brevifolia* bark (57).



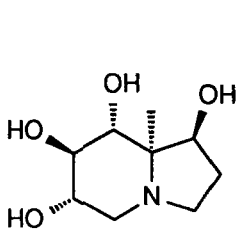




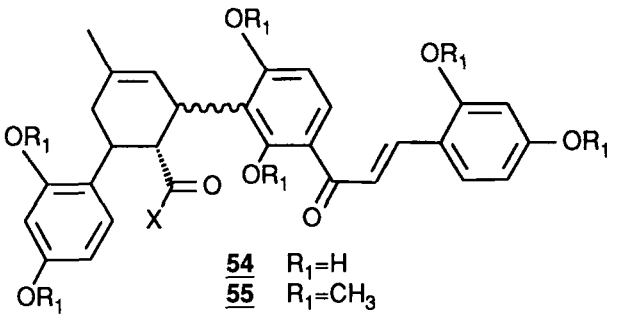
A method of evaluation of plant extracts for sweetness using the Mongolian gerbil has been described (58). Repin (**51**), a sesquiterpene lactone form Russian knapweed, *Acroptilon repens*, has been implicated (59) as the causative agent of nigropallidal encephalomalacia in horses, a disease characterized by necrosis and softening of the brain tissue. Significantly, the isomer epimeric at C-17, subluteolide is 4x less active in assays involving chick embryo sensory neurons. Compounds lacking this side chain exhibited much reduced activity. The acetogenins from the Annonaceae with a broad range of potent biological activities (cytoxicity, antitumour, antimalarial, antimicrobial, immunosuppressant, antifeedant and pesticidal) have been reviewed (60). A further *Phyllanthus* glycoside, phyllanthostatin 6 (**52**) has been isolated from *P. acuminatus* (61). The homoaporphine alkaloids have been reviewed (62).

A simplified plaque reduction assay for anti-viral agents from plant extracts has been developed and has allowed demonstration of the widespread and frequent occurrence of anti-viral activity in higher plants (63). Indolizidine and pyrrolizidine alkaloids, principally castanospermine (**53**) from *Castanosperum australe* and other plants, continue to attract great interest (64) because of their highly potent inhibitory effects on a variety of enzymes such as glucosidases (65,66), mannosidases (67), human immunodeficiency virus (HIV) (68), retroviruses (69) and tumour cells (70). The potential

for antiviral activities from plants has been reviewed (71). The first examples of truly natural Diels-Alder type adducts (such as **54** and **55**) from *Artocarpus* species has been reported (72). A review of iridoids has been published (73).

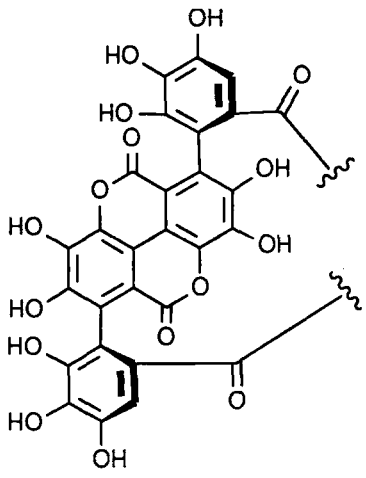


53

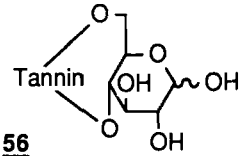


54 R_1=H
55 R_1=CH_3

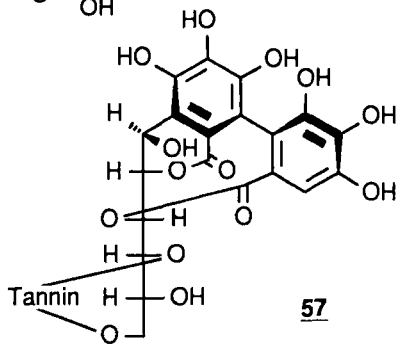
Tannins including both gallo- and ellagitannins have been shown to inhibit purified HIV-reverse transcriptase (74). These compounds, such as punicalin (**56**) and punicacortein C (**57**) shown ID_{50}s of about 5 μM. They may also inhibit HIV-replication in cell culture by additional or other mechanisms, probably by interfering with HIV-cell interactions.



= Tannin



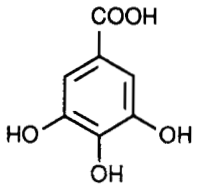
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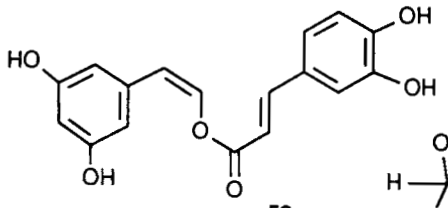
57

Digallic acid (**58**) and some derivatives have been shown to be inhibitors (K_i = 580 nM) of reverse transcriptase (75). **58** also inhibited DNA polymerases α and β but showed no effect on DNA polymerase γ, terminal deoxynucleotidyltransferase or *E. coli* DNA polymerase I (75). Further interest has been shown in complex tannins with some, such as woodfordine C (MW > 1000), a new cyclic dimeric hydrolysable tannin, from *Woodfordia fructicosa* leaves, demonstrating DNA topoisomerase II inhibitory activity (76). Potent inhibitors of xanthine oxidase such as **59** have been reported from the leaves of *Perilla frutescens* (77). The structure, distribution and biological properties of the oligomeric hydrolysable tannins have been reviewed (78).

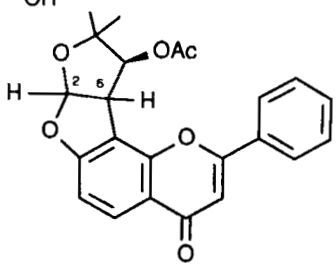
The differential facility with which chromone, chromanone and hydroxyflavones undergo microbial biotransformation has been interpreted in terms of a model for the interaction of the flavonoid nucleus with the *Aspergillus* hydroxylase (79). Chromone was not biotransformed whereas chromanone was converted to both chromone and chromanol; ring A hydroxylated flavones were hydroxylated at C-4'. A detailed investigation of protein-tyrosine kinase activity by 22 flavonoids has



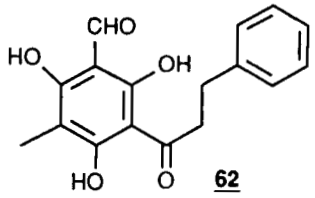
58



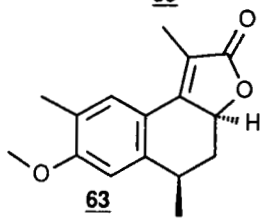
59



60 2,6-α
61 2,6-β



62



63

resulted in some structure/activity data (80). Flavonones and isoflavones are relatively inactive as protein-tyrosine kinase inhibitors but flavones or flavanols with hydroxyl groups on the phenyl ring were potent inhibitors (80). Further flavonoid molecules with biological activity has been isolated from the legume *Tephrosia semiglabra*: **60** and **61** show *in vitro* inhibitory effects on human platelet aggregation (81). A new chalcone (**62**) with antifeedant and antifungal activity has been isolated from *Psidium acutangulum* (82). Another compound with toxicity to fish is heritonin (**63**), a novel aromatic cadinane sesquiterpene from the mangrove plant *Heritiera littoralis* (83).

Conclusion - Since the last review of this subject in this series by one of us (84), natural materials have continued to be a fertile source of novel chemical structures possessing unique pharmacological activities. Although many of the natural products which are routinely discovered in screening programs do not afford drugs, either directly or by modification, many of them do become biochemical tools for mechanism of action studies, particularly as enzyme inhibitors or receptor ligands (85). In those cases where novel structures possess truly valuable biological activities without toxicity, the path to drug development is clear, such as in the cases of the immunomodulatory agent FK-506 or the CCK antagonist asperlicin. The marketplace success of natural products such as cyclosporin, avermectin and mevinolin has provided renewed impetus to the screening of plants, marine sources and microbial broths as sources of novel structures with new activities.

Biotechnology has also been increasingly incorporated into the drug discovery process: molecular biology can uncover new drug targets; recombinant DNA expression technology can provide those targets in quantity for screening; and genetic engineering can alter the organisms to mass produce compounds of interest (86,87). These advances, combined with new assay technologies which have made high-flux screening strategies successful, will only serve to increase the role of natural products screening in drug discovery, allowing the medicinal chemist to exploit the rich diversity of structure present in nature (88).

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Chapter 28. The Search for Molecular Diversity (II): Recombinant and Synthetic Randomized Peptide Libraries

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Introduction - Two new technologies for generating and screening large-scale peptide diversity have recently been described. The first of these, recombinant peptide diversity, allows the creation of very large numbers of randomly generated peptides using molecular biology techniques. These collections of peptides are screened to rapidly uncover those rare sequences, which might then serve as lead compounds for further development. The second approach, spatially addressable parallel chemical synthesis, allows the construction of arrays of well-defined chemical compounds, such as peptides. These arrays can be designed and screened with the goal of optimizing receptor binding. As with many of the tools of medicinal chemistry, these two approaches can be applied in a synergistic fashion to first find and then enhance lead compounds.

RECOMBINANT APPROACH TO GENERATING PEPTIDE DIVERSITY

Huge collections of peptides can be created by the molecular cloning and expression of random mixtures of oligonucleotides. Such collections might be put to many uses, provided clever strategies for efficient screening or selection are devised to identify the molecules of interest. For example, diverse populations of recombinant peptides have been used to analyze the properties of mitochondrial targeting and secretion signal sequences (1,2). In these studies, DNA sequences encoding signal peptides were replaced with random oligonucleotide sequences. Those clones that expressed functional signal peptides obtained a selective growth advantage. The peptide sequences were then quickly deduced from the DNA sequences of the recovered clones. A similar approach was used to study the catalytic site of β-lactamase (3,4). Portions of the β-lactamase gene were replaced with randomly synthesized oligonucleotides to produce large libraries of variants. These were screened for clones resistant to β-lactam antibiotics. Characterization of the resistant clones quickly provided a map of the tolerated amino acid substitutions and identified a number of mutants with interesting properties.

Recombinant libraries of peptides representing *limited* diversity have also served to identify ligands. Fragments of bacterial DNA were cloned into the expression vector λGT11, the plaque pattern was replicated by "lifting" onto membranes, and the membranes were probed for the presence of bacterial antigens with a panel of monoclonal antibodies (mAbs). By recovering and sequencing the DNA in the positive plaques, the relevant epitopes were identified (5). In a typical colony or plaque lift, the binding activity (protein) and the genetic information (DNA) are linked by their spatial address, thus allowing recovery of the DNA and identification of the active peptide. The use of plaque lifts places a practical limit of several million on the number of clones that can be efficiently screened.

The number of peptides, six residues or larger, that can be created by recombinant means is well beyond the reach of the most aggressive plaque screening schemes. There are over 10^9 (20^7) possible heptapeptides comprised of the genetically encoded amino acids. The development of "exposition" vectors has made practical the screening of collections of this size. These vectors display the peptides in a configuration available for affinity purification on immobilized binding proteins, while maintaining a linkage between the peptide and its encoding DNA. This arrangement allows the physical isolation of clones bearing active peptides and a means of rapid identification of those peptides by sequencing the DNA.

Several groups have constructed exposition vectors that display the peptides on phage particles (6,7), outer membrane proteins of bacteria (8-10), or bacterial surface structures such as flagella and pili (11,12). The most useful of these for ligand searching is a system that displays

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peptides fused to a coat protein of filamentous phage (6,7). The minor coat protein, pIII, normally mediates the infection of *E. coli* by the phage through binding to the pilus of the host cell. PIII is thus well adapted to interacting with structures external to the phage. Smith and coworkers showed that when DNA fragments encoding several peptides were inserted into certain locations in gene III, a fusion protein was expressed that retained the infective properties of pIII and carried the foreign peptides. Furthermore, these peptides were displayed in a way accessible to antibody binding. Subsequent refinement of the vectors and improvement of the affinity purification protocols provided a system capable of recovering phage that bear active peptides from a background of 10^6 to 10^8-fold excess of inert phage. The scheme of constructing and screening these libraries is illustrated in Figures 1 and 2. The effectiveness of this system for enriching peptide-bearing phage led to the concept of an epitope library: a random collection of peptides of six residues serving as epitopes for antibodies (7). An extension of this concept is the use of random peptide libraries to identify ligands to enzymes, receptors, and other biological targets.

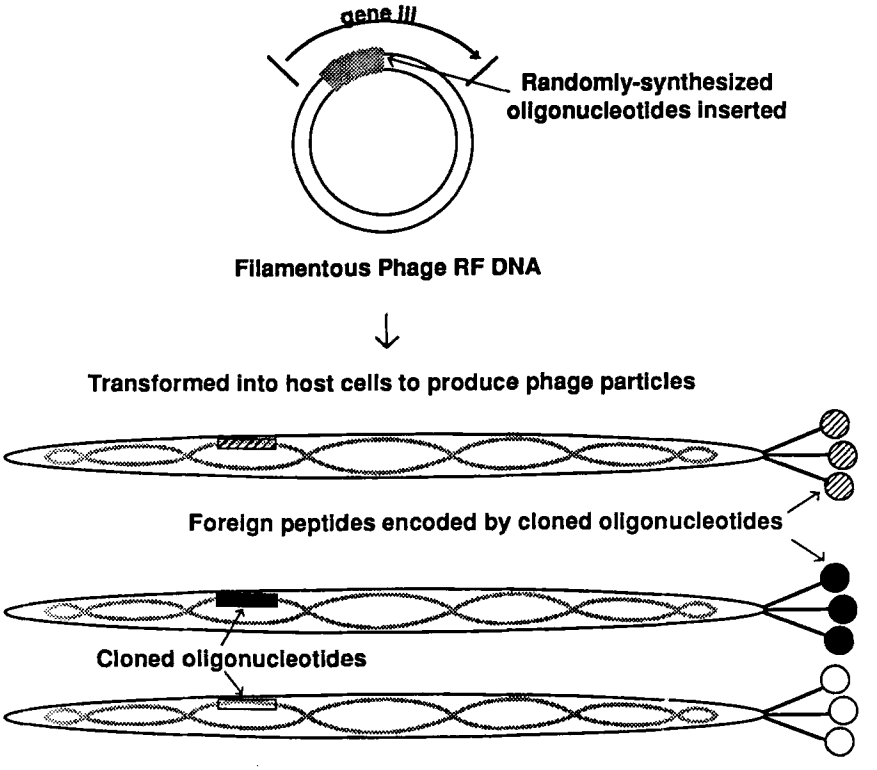


Figure 1. Construction of libraries of peptides expressed on filamentous phage. Mixtures of synthetic oligonucleotides containing random codons are ligated into a cloning site in the gene of the minor coat protein pIII. The recombinant DNA is transformed into an appropriate *E. Coli* strain, the library is amplified, and phage particles are isolated from the culture. The resulting library contains many phage, each expressing several copies of a single peptide on its surface.

The demonstration of the power of the phage exposition vectors coincided with the introduction of a high capacity transformation method that made practical the construction of plasmid libraries exceeding 10^8 recombinants (13). Three independent groups achieved the construction and screening of large, highly diverse peptide libraries to recover novel ligands (14-16). Scott and Smith constructed "epitope libraries" of hexapeptides fused to pIII at a site four residues from the

N-terminus (14). Libraries of 2.3 X 10^7 and 2 X 10^8 recombinants were constructed, and the former was screened against two monoclonal antibodies which recognize the same epitope on myohemerythrin. From among the population of peptides presented (statistically inferred by the authors to be about 10% of possible hexapeptides), a family of sequences was identified and verified to be ligands to the antibodies. Most of these peptides were closely related to the epitope (DFLEKI) and displayed tolerance of residue substitution consistent with a previous epitope analog study (17). Notably, one of the sequences recovered (CRFVWC) bore no discernible primary sequence similarity to the natural epitope. CRFVWC was shown to bind only to the higher affinity monoclonal antibody. In all, three of the recovered peptides contained pairs of cysteines and could be displayed in a disulfide bridged loop. The actual epitope, DFLEKI, was not recovered, a result explained by a statistical estimate of 94% that this sequence was not contained in the library.

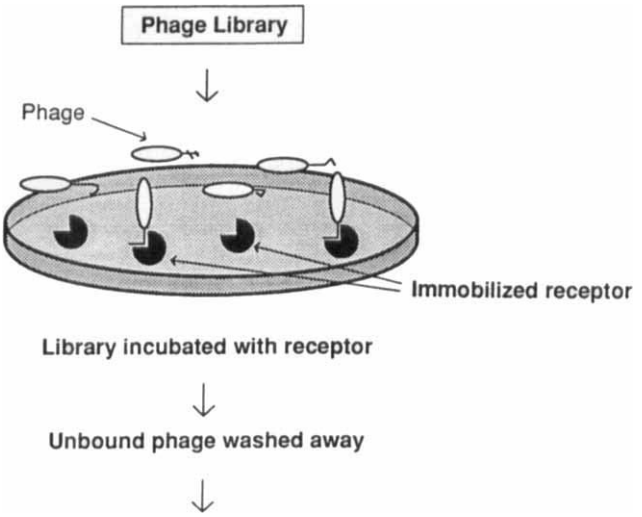


Figure 2. Screening ("panning") of the peptide library. The library of phage is incubated with receptors immobilized on a matrix. Phage carrying peptides that bind to the receptor are adsorbed and the inactive phage are washed away. The adherent phage are eluted, amplified in host cells, and reincubated with immobilized receptor. After several cycles of panning, the remaining phage are amplified and the DNA in the cloning site is sequenced, and the identity of the active peptides are deduced from the DNA sequences.

Devlin, *et al.* screened a library of longer peptides displayed on filamentous phage (15). This library contained 2.8 X 10^7 clones expressing random peptides of 15 residues. The peptides were located 2 residues from the N-terminus and were presented on a rigid linker of hexaproline. This library was screened against streptavidin, a "receptor" of biotin. They recovered 9 different clones that bound to streptavidin. These phage were significantly inhibited from binding to streptavidin in the presence of 1μM biotin indicating that the peptides bound at or near the biotin binding site. These clones shared a common tripeptide, HPQ, occurring in a variety of locations among the 15 variable residues of the 9 recovered phage. This may be the first description of a peptide ligand of streptavidin.

Cwirla, *et al.* described the construction and screening of a collection of 3 X 10^8 phage recombinants (16). These phage carried random hexapeptides displayed with free N-termini and attached to pIII *via* a diglycine linker. This library was screened against a monoclonal antibody (3-E7) that recognizes the N-terminus of β-endorphin (YGGFM) and cross-reacts strongly with leu-enkephalin (YGGFL) (18). A large number of peptide ligands were recovered. The 51 sequences explicitly identified bore a marked resemblance to the N-terminal residues of the immunogen (YGG), a key determinant of binding specificity for this mAb. A single duplication was found among

these 51 clones, suggesting the presence of several thousand different peptide sequences in the final enriched pool. Six of the 51 were chemically synthesized and their affinities for the antibody were determined. These six ligands proved to be of rather modest affinity (0.35 to 8 μM) compared to the known ligand leu-enkephalin (7 nM). The retention of peptides with affinities in the micromolar range, even after the extensive washing procedure used in the panning process, was an unexpected finding. This effect was likely due to multivalent interactions of the 4 or 5 copies of the peptides borne by each phage with the high density of immobilized IgG molecules. The multivalent effect allows the recovery of peptides with affinities as low as 10 μm, but prevents discrimination between peptides of modest and high intrinsic affinities. This group has subsequently shown that the few clones of nanomolar affinity can be recovered in preference to the abundant micromolar clones. This was accomplished by adjusting the wash time, temperature, and density of immobilized Fab. Most of the phage recovered with this protocol carried peptides with affinities for mAb 3-E7 estimated to be between 1 and 100 nM.

These reports demonstrate the usefulness of peptide exposition vector systems for finding ligands to binding sites without structural information about the binding site, the ligand, or the nature of their interaction. There are now additional examples of the use of these peptide libraries for the identification of continuous epitopes of antibodies. This system is also now being applied to the screening for ligands to pharmaceutically important receptors. New approaches to presenting the peptides and to building diversity into conserved structural domains should provide additional collections of potential ligands.

CHEMICAL APPROACHES TO GENERATING PEPTIDE DIVERSITY

An alternative to the generation of vast random peptide libraries is the more deterministic approach of directed synthesis and screening of specific peptide compounds. Compounds are synthesized one, or a few, at a time and then assayed for binding to a biological receptor. Determining the structure-activity relationship between a collection of peptide compounds and a particular receptor is then a highly repetitive, time-consuming process which involves synthesizing novel compounds, testing them for binding, redesigning compounds and retesting. A synthetic approach which is capable of generating a large, yet manageable, collection of peptides offers a viable alternative to iterative protocols.

Powerful approaches to simultaneous multiple peptide synthesis have been developed. A popular technique uses 96 well trays to achieve the parallel synthesis of 96 different peptides on "pins" (19). After synthesis, the peptides can be assayed while still attached to the pins, or cleaved and purified for later use. This method has been used very effectively to probe, for example, viral antigens for immunogenic epitopes (20). A different parallel peptide synthesis technique utilizes enclosed packets of resin to localize reaction products (21,22). Synthesis resin is sealed within reagent permeable bags, marked for bookkeeping, and then sorted into various reaction mixtures. The bags can then be batch mixed during amino acid coupling. The identity of the peptide within any particular bag is defined by the order of its exposure to the different amino acid coupling reagents. Preparative amounts of hundreds of different peptides can be synthesized simultaneously in this fashion. These technologies each rely on the need to physically segregate either the reaction reagents or products and therefore will be ultimately limited in the number of compounds that can be conveniently synthesized and assayed.

Light-directed, Spatially Addressable Parallel Chemical Synthesis - More recently introduced is a technology which greatly increases the number of peptides that can be synthesized and screened for receptor binding (23). The technique combines two well developed technologies: solid phase Merrifield chemistry and photolithography. The high coupling yields of Merrifield chemistry allow efficient peptide synthesis, and the spatial resolution of photolithography affords miniaturization. The merging of these two technologies is done through the use of photolabile amino protecting groups in the Merrifield synthetic procedure (24).

This technology is schematically illustrated in Figure 3. A synthesis substrate is prepared for amino acid coupling through the covalent attachment of photolabile nitroveratryloxycarbonyl (NVOC) protected amino linkers. Light is used to selectively activate a specified region of the synthesis support for coupling. Removal of the photolabile protecting groups by light (deprotection) results in activation of selected areas. After activation, the entire surface is exposed to the first of a set of amino acids, each bearing a photolabile protecting group on the amino terminus. Amino acid coupling only occurs in regions that were addressed by light in the preceding step. The solution of amino acid is removed, and the substrate is again illuminated through a second mask, activating

a different region for reaction with a second protected building block. The pattern of masks and the sequence of reactants define the products and their locations. Since this process utilizes photolithographic techniques, the number of compounds that can be synthesized is limited only by the number of synthesis sites that can be addressed with appropriate resolution. The position of each compound is precisely known; hence, its interactions with other molecules can be directly assessed.

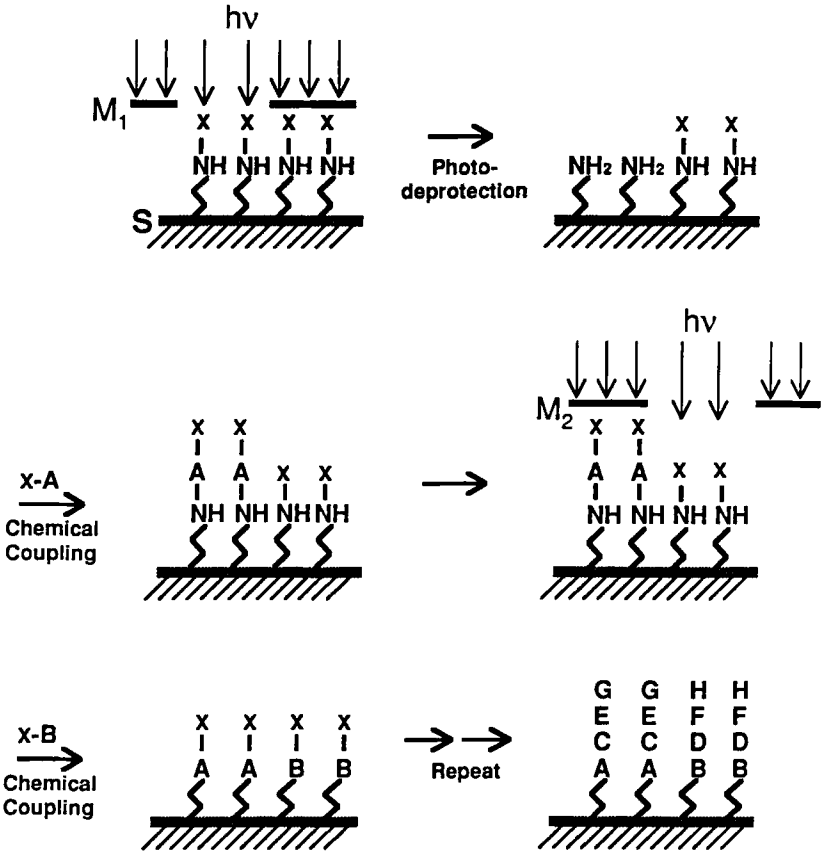


Figure 3. Light-directed spatially addressable parallel chemical synthesis. A substrate containing amino groups blocked by photochemical protecting groups is selectively illuminated through a mask (M_1). Spatially resolved activation of the substrate results from photodeprotection. The first of a set of building blocks (each bearing a photochemical protecting group X) is exposed to the entire substrate. Chemical coupling occurs only at those regions which were exposed to light in the preceding step. A different mask (M_2) is then used to selectively photoactivate a different region of the substrate. A second photoprotected group X-B is added and reacts at those sites addressed by M_2. The process is repeated to obtain the desired set of products.

Figure 4 shows a schematic of the light-directed synthesis of an array of the two peptides YGGFL and PGGFL. Following synthesis, the pentapeptide array was probed with the mouse Ab directed against β-endorphin (mAb 3-E7). This antibody requires the amino-terminal tyrosine for high affinity. A second incubation with fluorescein-labeled goat antibody to mouse IgG was used to label the regions containing bound 3-E7. The array was then scanned in an epifluorescence microscope.

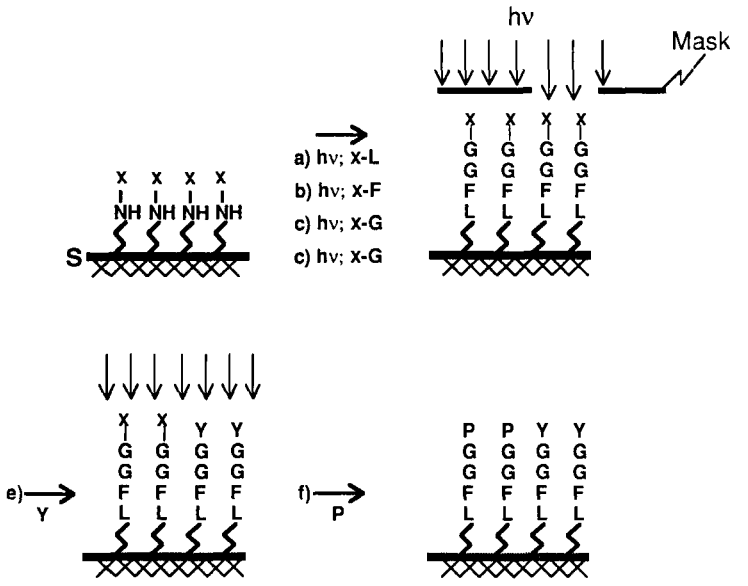


Figure 4. Light-directed synthesis of YGGFL and PGGFL. The entire surface was first activated by illumination (photolysis of all the NVOC protecting groups). Next, the HOBt activated ester of NVOC-Leu was allowed to react with the surface. After removal of the NVOC protecting groups from the Leu by uniform illumination, the substrate was treated with NVOC-Phe-OBt. Two repetitions of the cycle with NVOC-Gly-OBt generated a substrate containing NVOC-GGFL across the entire surface. At this stage, the substrate was illuminated through a 50 μm checkerboard mask. This effected localized photodeprotection of amino groups corresponding to the alternating light and dark regions of the checkerboard mask. The surface was then treated with Nα-tert-butyloxy-carbonyl-O-tert-butyl-L-Tyr. Finally, the surface was uniformly illuminated to photolyze the remaining NVOC-GGFL sites and treated with NVOC-Pro-OBt. After removal of the protecting groups, the surface consists of an array of Tyr-Gly-Gly-Phe-Leu (YGGFL).and Pro-Gly-Gly-Phe-Leu (PGGFL) peptides in 50 μm x 50 μm elements.

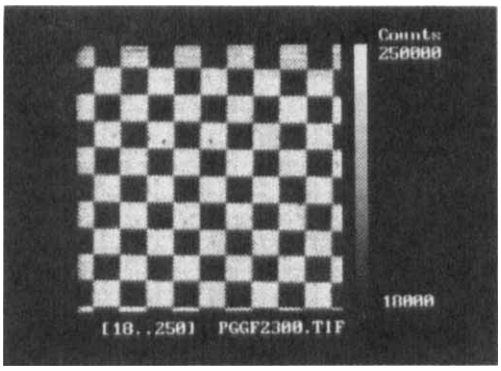


Figure 5. Fluorescence detection of the YGGFL/PGGFL checkerboard array. The array of peptides described in Figure 4 was incubated with 2 μg/ml mouse monoclonal antibody (3-E7). This was followed by incubation with fluorescein-labeled goat antibody to mouse to fluorescently label those sites with bound 3-E7. The surface was then scanned in an epifluorescence microscope (488 nm excitation). The fluorescence intensity was converted to a gray scale as indicated on the right of the Figure. The alternating bright and dark 50 μm features show that YGGFL and PGGFL were synthesized in defined locations according to the mask of Figure 4.

The result of the scan is shown in Figure 5. A high contrast fluorescence checkerboard image shows that YGGFL and PGGFL were synthesized in alternating 50 μm squares, that the YGGFL synthesized on the surface is accessible for binding to the antibody 3-E7, and that the antibody does not recognize PGGFL. The contrast between synthesis sites is very high at 50 μm resolution. This spatial resolution provides 40,000 discrete synthesis sites in a 1 cm by 1 cm square.

Combinatorial Synthesis Strategies - In a light-directed chemical synthesis, the products depend on the pattern of illumination and on the order of addition of reactants. As an example, an 8 step synthesis is shown in Figure 6a. The reactants are the ordered set {A,B,C,D,E,F,G,H}. In the first cycle, illumination through photolithographic mask M_1 activates the upper one-quarter of the synthesis area. Building block **A** is added and reacts only at the previously illuminated region. Illumination through M_2 activates the second one-quarter of the substrate, followed by reactant **B.** This process is continued through the first 4 cycles and results in the pattern of compounds shown in the first half of Figure 6a. Mask 5 then illuminates *across* the patterns generated in the first four cycles. This one illumination through mask 5 now generates 4 distinct dimers. The process is continued to generate the set of 16 compounds shown in the Figure. The final product set is {AE, AF, AG, AH, BE, BF, BG, BH, CE, CF, CG, CH, DE, DF, DG, DH}.

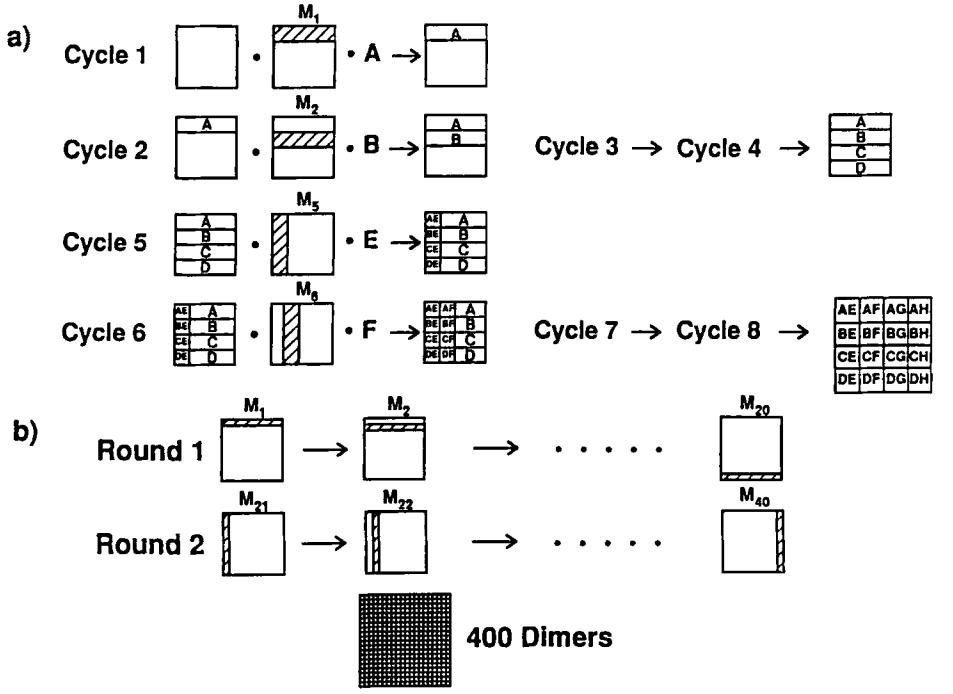


Figure 6. (a) Synthesis strategy used to synthesis {AE, AF,.AG, AH, BE, BF, BG, BH, CE, CF, CG, CH, DE, DF, DG, DH} from the chemical units [A,B,C,D,E,F,G,H]. (b) Extension of the synthesis strategy for the synthesis of all 400 dipeptides from a building block set of 20 amino acids.

This strategy can be applied to a large set of building blocks. For example, the complete set of 400 dipeptides can be formed from the set of 20 L- or D-amino acids. As shown in Figure 6b, the synthesis consists of 2 rounds with 20 coupling cycles per round. In cycle 1 of the first round, mask 1 activates 1/20th of the substrate for coupling with the first of 20 amino acids. An additional 19 illumination and coupling cycles are required to complete round 1. The substrate now consists of 20 rectangular stripes each bearing a distinct member of the 20 amino acid "building blocks".

The masks of round 2 are perpendicular to round 1 masks and, therefore, a single illumination-coupling cycle in round 2 yields 20 dipeptides. The other 19 cycles in round 2 complete the synthesis of 400 dipeptides.

A general formalism describes the combinatorial synthesis for any spatially addressable chemical synthesis. The process is conveniently expressed in matrix notation (23) and is based on the notion that at any given synthesis site the decision as to whether or not to add a monomer of the building block set is a binary process. If a particular position is to receive a new monomer group, it is addressed by a 1 (light on condition), if not, it receives a 0. For example, to form ACD from the ordered set {A,B,C,D}, the light switch consists of a vector (switch vector) [1,0,1,1]. Each product element is, therefore, given by the dot product of 2 vectors, a chemical reactant vector and a binary vector ([A,B,C,D] · [1,0,1,1] = ACD in the previous example). This formalism is very powerful in defining a complex chemical synthesis. For example, consider a 4 step synthesis with the order set of reactants A, B, C, D. Any switch vector consists of 4 bits for the 4 chemical reactants. There are 16 four-bit vectors [0,0,0,0] to [1,1,1,1]; hence, a maximum of 16 different products can be made from the ordered set of 4 reagents (one of the four compounds is the null or zero compound). For the example shown in Figure 6a there was an ordered set of 8 chemical reactants, and 16 compounds were formed. A more efficient synthesis strategy would create for all 2^8 (256) possible compounds. When we form the maximum number of compounds for a given number of chemical steps, we term the synthesis a *binary synthesis*. In a binary synthesis there are 2^n compounds for n chemical steps. Contained within the binary set of compounds, is the smaller set of compounds that would have been formed from the same ordered set of reactants by any other strategy. The only potential disadvantage of a binary synthesis is that the experimentally achievable spatial resolution may not suffice to accommodate all of the compounds.For the example shown in Figure 6b, we used 40 chemical steps to generate the 400 dipeptides. With the binary masking strategy we could produce over 10^{12} different compounds in the same number of chemical steps (this 10^{12} set of compounds would, of course, contain the 400 dipeptides). Practical limitations on spatial resolution, or the desire for a specific class of compounds, may require one to select a particular subset of the possible compounds for a given synthesis.

Detecting Molecular Interactions on the Array - The array of compounds can be assayed against a biological receptor. One convenient method is to incubate the array with a fluorescently tagged receptor (or receptor reporter molecule as was illustrated in Figure 5). A solution of the labeled receptor is applied to the synthesis array, incubated, then washed for a defined period of time. The fluorescence intensity at each synthesis site will depend on the affinity of the receptor for the compound, the concentration of receptor, and the number and density of interacting sites on the array. Other methods of reading the array are possible. Radiolabeled or chemiluminescent receptors could also be used, followed by appropriate detection. Characterizations other than binding might also be addressed in this format. For example, catalytic cleavage of a peptide at a site between a fluorescent donor or acceptor pair would lead to increased fluorescence. Another example is the identification of specific phosphorylation substrates by incubating an array with a specific kinase and radiolabeled ATP.

Synthetic Strategies for Ligand Discovery/Optimization - There are many potential synthetic strategies for ligand detection or optimization that could be used in a light-directed synthesis. One may choose to form the largest set of compounds for a given number of chemical steps, in this case, 2^n compounds can be formed in n chemical steps as described above. For a solid phase peptide synthesis of 16 couplings, 65,536 independent compounds are formed consisting of peptides from 1 to 16 residues (a mean length of 8). Alternatively, one could form all combinations of a set of chemical building block units for a given length. This simple synthesis format would generate X^l, where X is the number of chemical building blocks, and l is the length of the peptides. For X = 20 peptides (the L- or D-amino acids for example) and l = 2, the 400 combinations are formed. If this were extended to l = 4, the 160,000 possible tetrapeptides with a building block set of 20 is a tractable goal. The light-directed method is well suited for rapid generation of analogues of known ligands. For example, the peptides in the array might be stabilized to proteolysis by introducing D or other unnatural amino acids. The introduction of a variety of chemical buildings blocks into the synthesis can be used to confer desirable physical properties into the compounds on the array (e.g.hydrophobicity, charge character, conformational restriction, etc.). The number of analogues synthesized on the array is limited only by the spatial resolution. The present limit for high contrast photodeprotection is about 20 μm which can yield densities of 250,000 compounds in 1 cm by 1 cm.

INTEGRATED APPROACH TO LIGAND DISCOVERY AND OPTIMIZATION

The recombinant and light-directed approaches have distinctive strengths and are highly complementary technologies. In a coordinated discovery project, a peptide ligand identified by the recombinant method serves as a starting point for derivatization with the synthetic method. In order to explore this complementarity, results from screening the fd phage recombinant library against antibody 3-E7 have been used to design a 1024 compound array for synthesis by the light directed method. The results from initial panning of the phage hexapeptide libraries gave the following trends: 100% of the sequences began with Y; over 90% had G in the second position; 40% had either G or A in the third position; 93% of the residues in position 4 were the four large hydrophobic residues F, L, I, W; the last two positions were virtually random (16). We therefore designed the synthesis of the following ten step binary synthesis (1024 peptides): (f+0)(Y+0)(G+0)(A+0)(G+0)(T+0)(F+0)(L+0)(S+0)(F+0) (where f is D-phenylalanine) (21). This synthesis generates fYGAGTFLSF as the longest peptide. All of the corresponding shorter deletion peptides are synthesized on the array as well.

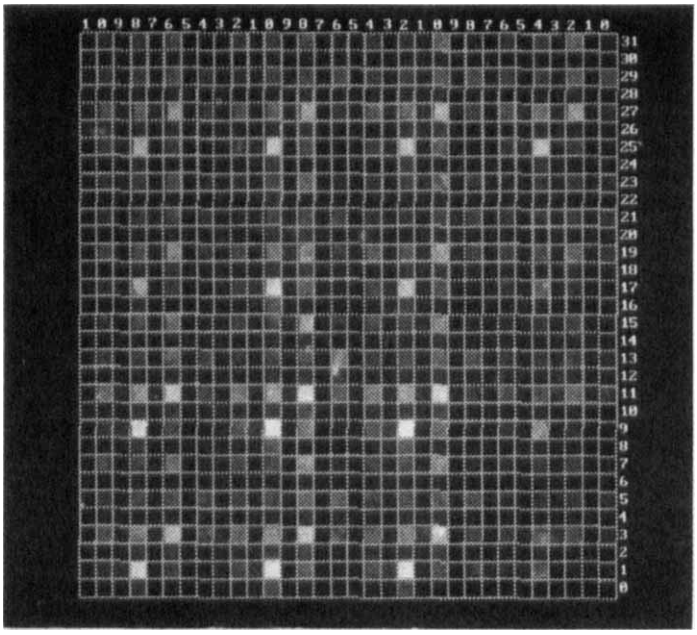


Figure 7. Fluorescence scan of an array of 1024 peptides generated by a ten-step binary synthesis. Following synthesis and deprotection, the array was fluorescently stained as described in Figure 5. The fluorescence intensities range from 3,000 to 22,400 counts. The polynomial expression (23) for this synthesis is (f+Ø)(Y+Ø)(G+Ø)(A+Ø)(G+Ø)(T+Ø)(F+Ø)(L+Ø)(S+Ø)(F+Ø). The coordinates for each peptide are indicated. The identity of each peptide is provided in ref. 23.

The results of a fluorescence scan after antibody labeling of the array is shown in Figure 7. The scan shows a range of fluorescence intensities ranging from 3,300 (background counts) to 22,400 counts. Of the 1024 compounds, only 15 exhibit an intensity greater than 12,300 counts. These are: YGAFLS, YGAFS, YGAFL, YGGFLS, YGAF, YGALS, YGGFS, YGAL, YGAFLF, YGAF, YGAFF, YGGLS, YGGFL, YGAFSF, and YGAFLSF (23). These high intensity peptides exhibit the following trends: all begin with YG; only G or A appear in position 3; F or L dominate at position 4. Additional synthesis designed against 3-E7 have shown that YGAFMQ, YGAFM and YGAFQ give stronger fluorescence intensities than does YGGFM, which is more closely related to the immunogen β-endorphin. The results from this experiment are in striking agreement with the results from the recombinant approach.

These experiments illustrate the complementarity between the recombinant and light-directed synthesis approach. Once a ligand is identified with the phage library, parallel chemical substitution can be used to systematically investigate chemical characteristics affecting binding. Iteration between the two technologies is possible through the careful design and construction of both peptide libraries and synthetic strategies. Recombinant peptide diversity and light-directed parallel chemical synthesis provide new tools for ligand discovery and optimization and are now in use for drug discovery.

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Chapter 29. Receptor Modeling by Distance Geometry

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Introduction - Distance geometry is a general molecular model-building method, best known for determining three-dimensional solution structures of peptides, proteins, and nucleic acids from nuclear overhauser effect (NOE) distance measurements (1,2). Distance geometry has also been applied in a variety of ways to build models of proteins and receptors and to model the interaction of small molecules with receptors. Ghose and Crippen recently published a comprehensive review of distance geometry and its application to receptor modeling, emphasizing their distance geometry quantitative structure-activity relationship (QSAR) approach (3). We will focus on additional distance geometry approaches which have application to receptor modeling and only briefly describe the distance geometry QSAR method.

Distance geometry converts a set of distance bounds (minimum and maximum allowed distances) into a set of coordinates consistent with these bounds (4,5). A molecular structure is described by the set of all pairwise interatomic distance bounds in a distance bounds matrix. This distance bounds matrix describes the complete conformation space of a molecule by entering the maximum possible distance (upper bound) between each atom pair in the upper diagonal and the minimum possible distance (lower bound) in the lower diagonal. All possible conformers therefore must lie between these upper and lower distance bounds. Additional distance bounds constraints, for example from NMR data or a proposed pharmacophore model, may be added to the distance bounds matrix. Distance geometry converts this usually underdetermined distance bounds information into randomly sampled sets of three dimensional coordinates.

Protein Structure Modeling - O'Neil and DeGrado used distance geometry to predict the conformation of a calcium-binding loop of calmodulin, as part of their attempt at predicting the entire calmodulin structure based on the homologous sequences and X-ray structures of intestinal calcium-binding protein (ICB) and carp parvalbumin (6). The calmodulin sequence had two insertions in one of the ICB calcium binding loops. Distance geometry was used to generate models for the loop by constraining the ends of the loop to the ICB X-ray coordinates, forcing the side chains of four polar residues and a main chain carbonyl to interact with the calcium cation in a geometry identical to that from the second ICB loop, while maintaining octahedral coordination about the calcium. The remainder of the loop was allowed to remain flexible. Ten unique loop conformers that satisfied the constraints were identified from thirty random trials; these ten structures were energy refined with molecular mechanics. One of the refined conformers had reasonable contacts with the rest of the calmodulin model when reinserted back into the complete protein structure.

Havel and Snow used distance geometry to build complete protein models (not just loop regions) of unknown structures based on the sequence alignments and X-ray structures of homologous proteins (7). Sequence alignments are converted into distance and chiral constraints to maintain equivalent secondary structure, hydrogen bonds, and disulfides. Although the approach still depends critically on correct sequence alignment, the model-building procedure is automated and less biased than conventional approaches, resulting in an ensemble of random models which are consistent with the constraints implied by the sequence alignment. Several test cases demonstrated the feasibility of the approach, but no comparison with other methods was made.

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Drug-Receptor Docking - Ripka and coworkers (8) designed phospholipase A_2 (PLA_2) inhibitors based on the high resolution X-ray crystal structure of bovine pancreatic PLA_2 (9). Potential binding modes of the substrate phospholipid were first modeled in the active site to suggest conformationally restricted mimics. A model was proposed in which the hydrated ester carbonyl intermediate was hydrogen-bonded to the active site His-48, and a phosphate oxygen was located at one of the water positions in the coordination sphere of the calcium. Distance geometry was used to generate many potential phospholipid binding modes with these constraints. The substrate fits generated by distance geometry suggested a hydrophobic groove in the PLA_2 active site which could accept a naphthalene ring. This offered the possibility of building a rigid framework from which additional functionality could be directed to interact with other parts of the site, resulting in the design of a series of acenaphthene inhibitors with good *in vitro* activity. Distance geometry was also used to dock the resulting designs into the active site, with no constraints except to maintain reasonable minimum intermolecular distances, to determine additional potential binding modes by randomly sampling the inhibitor designs' conformations within the confines of the active site.

Pharmacophore Modeling using the Ensemble Approach - A problem that often occurs in molecular modeling of bioactive compounds is to define the geometry adopted by a pharmacophore. A pharmacophore is a set of atoms or groups which are present in each bioactive molecule and which are required for activity. Often the assumption is made that molecules which have the same biological activity interact at the same receptor by presenting the pharmacophore elements to the receptor in the same way. The question, especially in the absence of a three dimensional structure for the receptor, is whether or not a unique three dimensional geometry can be inferred for the pharmacophore elements by examining several different molecules. In other words, are there conformations for each molecule in which the corresponding pharmacophore elements have the same geometrical arrangement?

The important concept is that, while each molecule considered by itself may be quite flexible, the assumption that certain pharmacophore elements have the same geometry between different molecules introduces new constraints that may restrict the allowable conformational space available to each molecule. The computational problem can be stated explicitly in the following way: generate conformations for each molecule such that certain atoms or other structural elements (such as lone pairs or ring centroids) can be overlapped with the corresponding elements in each of the other molecules. This can be expressed quite naturally in a distance matrix. If several independent molecules are placed into a distance matrix representation, the intramolecular distance bounds for each would be present but all intermolecular distances would have lower bounds of zero and upper bounds of infinity. Applying the distance geometry algorithm to such a distance matrix would result in conformations for each molecule in random orientations relative to the other molecules. To force certain atoms from each molecule to overlap, the intermolecular upper bounds between those atoms must be lowered to a small value (say 0.3 Å) that represents the tolerance in the overlap. Then the distance geometry algorithm will generate conformations for each molecule such that the pharmacophore elements overlap between molecules. This is the essence of the ensemble distance geometry method (10).

For example, the nicotinic agonists shown in Figure 1 were used (along with two others) in an

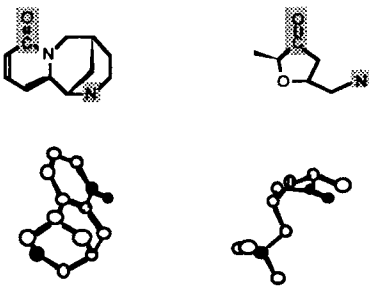


Figure 1

ensemble distance geometry calculation in which the corresponding highlighted atoms were used as the pharmacophore (10). The resulting geometry is shown in the lower three dimensional views with the pharmacophore atoms in dark filled circles.

Wong and Andrews used manual atom-by-atom superimposition, electrostatic potential map superimposition, and a systematic torsion search method to propose a model for the binding of convulsants to the picrotoxinin site of the GABA receptor (11). A total of ten superimposition models were found by these approaches. Ensemble distance geometry also found these ten models, plus one additional model which was not found by the other approaches.

There are several possible outcomes for such a calculation. First, there may be no acceptable solution. Second, there may be only one solution in that there is only one possible pharmacophore geometry and each molecule has only one conformation which can present that pharmacophore geometry. Third, there may be only one pharmacophore geometry but multiple conformations of at least some of the molecules which are compatible with that geometry. Finally, there may be many solutions both for the pharmacophore geometry and some or all of the molecules.

Systematic torsional search methods have also been used to determine pharmacophore geometries (12,13). The method essentially performs a complete systematic torsion conformational search on one molecule and then uses the resulting sets of distances between pharmacophore elements to prune the search tree for the next molecule, and so on. After all molecules are searched, the remaining sets of distances represent those which can satisfy pharmacophore geometries which are common to all compounds. By recording the torsion angles, the conformations of each compound can be regenerated.

There are advantages and disadvantages to each method. The computational complexity of the ensemble method is a polynomial function of the number of atoms rather than an exponential function of the number of rotatable bonds. Therefore, for systems with many rotatable bonds, the ensemble method will be faster. However, pruning of the search tree as early as possible can improve the speed of the systematic search approach (12). The ensemble method handles ring systems naturally and thus can alter ring conformation in the course of searching for pharmacophore geometries. Torsional searches have difficulty altering ring conformations since changing a single torsion within a ring causes distortions of bond distance and angles in other parts of the ring system.

If the chirality of some centers is unknown, the ensemble method can allow the chirality at those centers to randomly vary and choose the chirality which is compatible with the pharmacophore geometry. Systematic torsional searches would need to be done with all possible chiralities of unknown centers to ensure that solutions were not missed.

The main disadvantage of the ensemble method relative to torsional searching methods is that the ensemble method produces random solutions rather than being more deterministic. For a given angle step size, the systematic torsional search assures that all possible solutions have been examined. However, computer time constraints often force one to choose a relatively large angle step size (for example 30°) and there is no guarantee that solutions are not missed because they fall between the steps. In addition, in pharmacophore modeling applications, one is usually not interested in enumerating all possible solutions. The useful results are: no answers, in which case the problem has probably not been correctly formulated; one answer, which might be the unique pharmacophore geometry; or more than one answer, in which case the problem is underdetermined and more data are necessary.

The ensemble method (and distance geometry in general) also cannot naturally handle problems in which constraints are phrased in terms of lists of acceptable torsion angles (*i.e.* that an angle can be either *trans*, *gauche* +, or *gauche* -). However, the useful special case of *cis* or *trans* (for example, peptide bonds) can be handled by distance geometry using chiral constraints to keep the bonded atoms planar.

Distance Geometry QSAR - Crippen applied distance geometry to the problem of three-dimensional receptor mapping (3). Ghose and Crippen recently reviewed the approach in detail (3). A very similar approach was also reviewed (14). Their method proposes the geometric requirements of the

receptor site based on the experimental data of binding affinities of a series of ligands, which may be conformationally flexible, and hypothesized binding modes (*i.e.* pharmacophore model) for each ligand. The result is a low-resolution, three-dimensional model of the receptor binding site, which is described as a series of points in space (site points) that interact with specific ligand atoms or groups of atoms (ligand points). Each ligand point is described by atom-centered physicochemical properties (molar refractivity, hydrophobicity, and partial charge) (15,16). A specific interaction 'energy' is assigned to each site point - ligand point interaction by a modified quadratic programming optimization procedure, yielding a quantitative prediction of the binding affinity of each ligand to the site model. Optimization of interaction parameters is performed under the constraint that the interaction energy of the proposed binding mode is more favorable than the interaction energy of all other geometrically possible binding modes. Optimization continues until the proposed binding mode is either proved to be the best or found to be inconsistent, when it is replaced by the most favorable binding mode encountered so far, and optimization is continued. This approach avoids the temptation to bias the fits and avoids preconceptions on how a particular ligand might bind. The three-dimensional geometric model of the binding site involves both energetic and steric features. Since the approach predicts the mode of binding as well as the interaction energies it offers the opportunity to suggest conformationally restricted analogs that might maximize binding to the site. Ghose and Crippen generated a model for the inhibition of *E. coli* dihydrofolate reductase by 39 compounds from two different structural classes (17). The model contained 19 site pockets of 9 different types and qualitatively agreed with the X-ray crystal structure of the enzyme's active site.

Ghose and Crippen recently modified their approach to eliminate much of the subjectivity previously required in choosing conformations and binding modes (18-20). A conformational search is performed for each molecule to identify the lowest energy conformers. A priority score is assigned to each conformer based on the sum of the experimental free energy of binding of the molecule to the receptor site and the calculated energy of the conformer relative to the global energy minimum for the molecule. Starting from the highest priority conformation, the low energy conformers for the remaining molecules are superimposed on it to give the best overall match based of the atom-centered physicochemical properties. This approach is performed for several different high priority reference conformations, each producing a different possible superimposition model. Each model is then evaluated in the remaining steps to determine the best model. Site points are initially placed at the positions of superimposed atoms and remaining non-coincident atoms. This results in a large number of site points, which is reduced using stepwise reverse regression to eliminate insignificant site points. The optimization step is similar to their original approach. This modified approach appears to be a significant improvement and has been used to model the binding of 28 antiviral nucleosides against an unknown receptor of parainfluenza virus (18), the binding of 29 benzodiazepine receptor ligands to the benzodiazepine receptor (19), and the antileukemic activity of 21 purine-6-substituted nucleosides (20).

Voronoi Binding Site Models - Recently, a new method has been developed which produces a novel receptor site description, called a Voronoi binding site model, using information from the structures and binding affinities of a series of ligands (21,22). The binding site is described by dividing space up into a set of Voronoi polyhedra, which are derived from a set of 'generating points' in a simple way: each polyhedron consists of all points which are closer to one generating point than to any other. Thus, each generating point results in one Voronoi polyhedron. A binding site model is specified by the number and location of a set of generating points. In addition, each Voronoi region is assigned a set of interaction parameters for each type of ligand atom. Thus, a ligand can be placed into the receptor model and an interaction energy calculated by summing the interactions of each atom with the region that contains it. For example, Figure 2 shows a simple receptor model with two regions. Note that there are many equivalent ligand binding modes. The only aspect which is important for interaction energy calculations is the region into which each ligand falls.

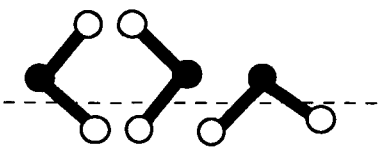


Figure 2

The object of the Voronoi binding site approach is to find a set of generating points and interaction potentials which reproduce the experimental binding constants within experimental error when each ligand is bound to the receptor site model in the most favorable way. The specific steps in developing a model are: (i) summarize the conformation space of each ligand; (ii) propose a site geometry; (iii) determine all geometrically allowed binding modes of each ligand; (iv) determine the interaction parameters. If step (iv) fails, then the method starts again at step (ii) with a new site geometry.

The advantages of the Voronoi method are that it does not require the *a priori* assumption of a pharmacophore, it accounts in a quantitative way for the observed data, and it does not impose any more detail on the binding site model than is necessary to account for the data. On the other hand, it is necessary for the user to propose the binding site geometry and the overall process is computationally expensive. The method has been successfully applied to two fairly simple test cases (22,23), but at this point it is not yet clear how difficult larger problems will prove to be.

Summary - Relatively few applications of distance geometry have appeared since its original application to chemical structure problems by Crippen over ten years ago (24). The vast majority of applications and publications have focused on determining solution structures of small and macromolecules from NMR experiments (1,2). Distance geometry software is now more readily available (25-27), along with a steadily increasing number of applications and publications which demonstrate its unique ability to generate molecular models for complex situations involving drug-receptor docking (8), pharmacophore modeling, protein structure prediction (6,7), and conformational analysis (28,29).

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Chapter 30. Sequence-defined Oligonucleotides as Potential Therapeutics

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Introduction: - The sequence specific modulation of gene expression within a cell using oligodeoxynucleotide (ODN) analogs is a significant approach to potential therapeutics. This field, traditionally called antisense RNA because of the intent to down-regulate expression of a disease-causing protein by inhibiting the translation of its precursor messenger RNA, must be modified to include cellular genomic duplex DNA as a target. This review focuses on recent work on both the inhibition of gene expression using ODNs by mRNA hybridization via Watson Crick base pairing and the inhibition due to triple helix formation with duplex genomic DNA. The antisense RNA and duplex targeting triple helix approaches share common features not usually required of drugs in an absolute sense: stability in serum and in tissue; access to intracellular targets; and ability to attain significant concentration levels in the cytoplasm and nucleus. These stringent pharmacokinetic and pharmacodynamic requirements will be discussed in context of the antisense approach.

ODN mediated antisense RNA has been the focus of several recent reviews (1-4). Triple helix inhibition of gene expression has not been reviewed, however, and consequently will receive more extensive treatment here. Other classes of ODN-mediated modulation of gene expression show therapeutic potential. Preliminary studies have demonstrated titration of transcription factors from their genomic DNA by using double-stranded ODN sequences identical to the genomic sequences bound by these factors (5,6). Both cases targeted positive regulator proteins which resulted in suppression of gene expression. Yet another approach for using sequence-defined oligonucleotides as potential therapeutics is via binding to proteins (7) and to small molecules (8). These promising approaches are in their infancy and will not be discussed further.

DOUBLE-STRANDED DNA TARGETING BY TRIPLE HELIX FORMATION

Research in the area of triple helix nucleic acid structures includes extensive studies on both homopolymer sequences and alternating copolymers (9-11). Recent work has shown the sequence specific recognition of various polypurine tracts using Hoogsteen base pairing (12-15) and a deoxyguanosine (dG) driven binding mode (16). Most work, however, centers on *in vitro* binding data and thus far there is a paucity of biological results. The methods for analyzing binding affinity and biological data will be discussed after presentation of the principles of triple helix binding and how it has been improved.

The Hoogsteen binding motif requires a polypyrimidine ODN which is parallel to the targeted purine strand in the duplex. Recognition requires protonation of the deoxycytidines (dC) in the ODN in order for efficient pairing with G in the target purine strand (Figure 1) (12). This binding motif has been enhanced and extended in a number of ways. 5-Methyl-dC used in place of dC

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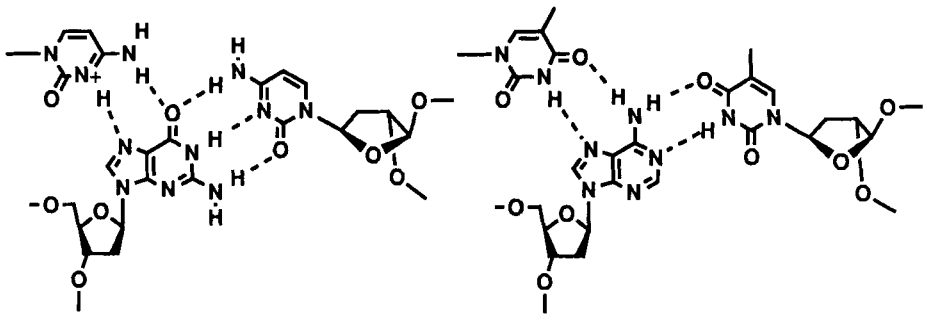


Figure 1. Triple helix geometry by Hoogsteen binding of a T to an A-T basepair and of a protonated C to a G-C basepair.

results in enhanced binding, allowing *in vitro* duplex recognition to occur at neutral pH (17,18). The pH sensitivity of triple helix formation is not diminished by such a substitution, not surprising in light of the similarity of pKa values of the 5-methyl-dC and dC (19). The extreme specificity of Hoogsteen triple helix interactions has been shown by the largely single site recognition in a 340 kilobase yeast chromosome (20). The triple helix recognition code, usually limited to polypurine tracts, has been partially extended by using G in an ODN to tolerate a thymidine (T) in the midst of a polypurine target tract (21). Another mechanism for code extension is the utilization of an alternate strand approach (22). This involves recognizing a polypurine tract in standard fashion with an ODN and then crossing the major groove of the duplex and recognizing an adjacent polypurine tract on the opposite strand. Such an ODN consists of two 5' to 3' polypyrimidine sequences joined at their 3' ends with an appropriate linker. In the published case, the linker was a 1,2-dideoxyribose derivative.

The G driven motif does not require protonation of any heterocycle for recognition, and consequently binding is likely a pH independent event. The code for this form of recognition is somewhat ill defined, however. Work reported in the recent patent literature claims that binding can occur with the ODN having a polarity parallel to the targeted purine strand of the duplex or antiparallel depending on the sequence (23). In the ODN dG is used to recognize G in the

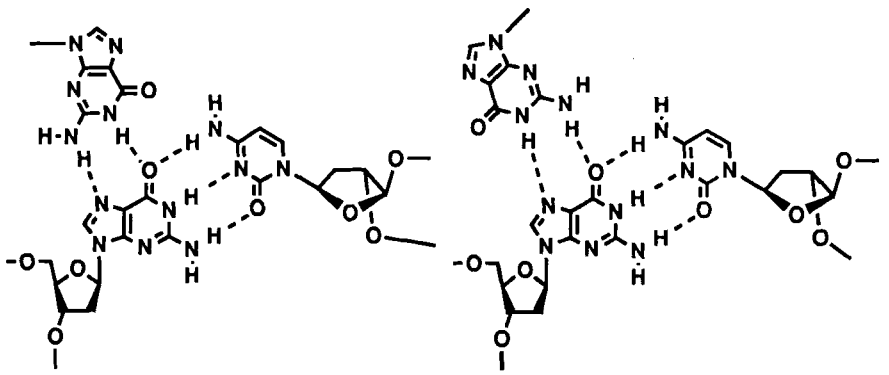


Figure 2. Two possible modes of G-GC binding.

target; two possible modes of binding are shown in Figure 2. However, for the recognition of an adenine (A) in the target, dA or alternatively T can be used in the ODN (23).

Crosslinkers and Intercalators - One approach to ultimately enhance a biological effect of a triple helix complex is the formation of a covalent crosslink. Attempts have focused on photochemically triggered reactions and alkylation events targeting the N^7 of a specific G in the target duplex. Although photochemical crosslinking is a low efficiency process (24,25), alkylation approaches have shown higher efficiency (26-28). To date, none of these approaches have demonstrated in the published literature an enhanced biological effect on the inhibition of transcription or replication either *in vitro* or *in vivo*.

Another approach to enhance binding is the attachment of appending groups which stabilize the triple helix complex. Intercalating agents have found extensive use in the field of duplex stabilization in the targeting of mRNA (4). This has been extended into the triple helix field, where an acridine derivative on the 5' end of a oligopyrimidine has been shown to enhance triple helix stability (29).

Phosphate and Ribose Modifications - The effects of modified phosphate linkages and ribose sugars on duplex formation have been well studied (2). However, little structural work in this area has been applied to the triple helix area. Methylphosphonates (MP) have undergone intensive study as phosphate analogues in the antisense RNA area. This linkage is only now being critically evaluated in regard to ODNs substituted with MP for their ability to form triple helix structures with native duplex target. At present only homopolymer sequences have been examined and triple helix formation was not observed (30).

The effects of modification of the ribose sugar on triple helix formation has also received attention. The alpha anomers of nucleosides have been incorporated into ODNs and have been shown to form triple helix structures with native duplex DNA (24,25). The polarity of the ODN binding in these cases is ambiguous, with parallel binding observed in some sequences and antiparallel in others. Structurally, the complexes are unique and such modified ODNs have been shown to be stable to nucleases (31).

Cleaving Agents - The desire to create artificial sequence specific nucleases with high turnover numbers and which have the potential to function *in vivo* is an intriguing topic (32). To date many moieties have been attached to ODNs to confer a nuclease function. The first demonstration in the triple helix area was the use of iron(III) EDTA as a free radical generator under the appropriate redox conditions (12). Since then, other redox-activated transition metal complexes have been investigated, such as phenanthroline (33,34) and porphyrins (35). These redox-activated systems are a powerful assay tool for triple helix formation, but their suitability for *in vivo* applications is questionable. Most have a relatively low cleavage efficiency, and actual catalytic turnover has not been demonstrated. Additionally, for *in vivo* applications, the redox activation must occur in the nucleus of a living cell, suggesting that chemical nucleases may be inadequate. Photochemical cleavage has also been demonstrated in triple helix and again the efficiency of reaction is low (36). Another approach is the coupling of a triple helix forming ODN to DNase I (37). This semisynthetic enzyme is capable of sequence-specific triple helix-mediated cleavage of duplex DNA, but its use will be limited to *in vitro* applications.

In Vitro Analytical Methods - The analysis of *in vitro* sequence specific triplex formation requires reliable analytical methods and a variety of techniques have been applied to the problem. The first technique utilized the iron EDTA redox cleavage system (12). Triple helix formation was visualized by the formation of triple helix on a radiolabeled duplex target and redox activation to produce local single stranded cleavage followed by denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiography. This assay also allows for the assessment of the orientation of the third strand relative to the purine duplex strand, i.e. parallel versus antiparallel. Variations of this method have been attempted using other cleavage agents (4).

Another powerful technique is the footprint (38), which involves forming the triple helix with a radiolabeled duplex target and then performing a limited digest with a DNA cleavage agent, such as DNase I or copper phenanthroline, followed by denaturing PAGE and autoradiography. Triple helix formation results in protection from digestion in the region of the DNA duplex and can be easily observed. A variation of this technique involves using triplex formation to protect or cover a restriction enzyme site, followed by use of the restriction enzyme as cleavage reagent (39,40).

The gel shift assay has been used extensively in protein-DNA interaction studies. This technique has been applied to a limited extent in the triple helix area (16,41), and this involves radiolabeling the triple helix-forming ODN, followed by forming triple helix with the target duplex. Electrophoresis under non-denaturing conditions results in the single-stranded ODN shifting to a slower mobility band upon complexation with the duplex target. A titration using increasing concentrations of duplex allows for an assessment of binding constants. A requirement in this assay is that the triple helix complex is stable to the conditions and time of electrophoresis.

Thermal denaturation of complexes is also a standard technique for the analysis of duplex formation, and this technique has recently been applied to sequence specific triplex interactions (19,41-44). In these cases, as with the study of duplex interactions, complexes show a hypochromicity relative to the unbound states. This results in a plot of UV absorbance versus temperature from which melting temperatures and thermodynamic values can be determined.

2D NMR techniques have matured in recent years enough to allow the solution structural determination of biomacromolecules, including triple helix structures (45-47). These NMR experiments have confirmed Hoogsteen base pairing of the third strand and protonation of the dC at N^3. Such 2D NMR techniques hold promise for more detailed structural information. X-ray crystallography also offers tremendous potential for understanding triple helix structures at high resolution. At present, only X-ray studies on fiber diffraction of homopolymer triplex helices have been published (48).

Biological Effects - The ultimate goal for ODN research is the development of therapeutics, but demonstration of any *in vivo* biological effects with ODN has been slow. Triple helix formation in tissue culture may have been demonstrated once (49). In this example, inhibition of replication of SV40 in COS cells was observed upon the addition of ODN octathymidine bearing an acridine moiety on the 5' end. This ODN was targeted to a 8mer polyadenosine tract in the origin of replication of SV40. Careful controls were devised to exclude trivial explanations such as ODN degradation and acridine release. The antiviral effect was clearly

due to the ODN, but confirmation that the effect was mediated by an intracellular triple helix event requires further study.

Another study, which approximates a biological effect, demonstrated the inhibition of transcription of the myc oncogene in an *in vitro* system (16). The inhibition of DNA binding proteins by triplex formation has also been demonstrated *in vitro* (50). The paucity of biological effects attributable to ODN-mediated triplex formation presents a formidable challenge for the future: a clear demonstration of ODN triple helix formation with a genomic target inside of a living cell and subsequent inhibition of specific gene expression.

mRNA TARGETING BY DUPLEX FORMATION

Permeation - The application of ODNs both for targeting DNA by triplex formation and for targeting RNA require intracellular localization of free ODN. Permeation of ODN through cellular membranes is a very inefficient process due to the highly charged character of the phosphates at neutral pH. Consequently, permeation is recognized as a major hurdle for the practical application of ODNs as drugs.

Previously, a large number of ODN conjugates were prepared to enhance cellular uptake of ODN (1). Some of these conjugates showed enhanced biological activity relative to the unconjugated ODNs. Attachment of hydrophobic chains to ODN led to activity against both vesicular stomatitis virus (VSV) and influenza virus at concentrations where the unconjugated ODNs were inactive (51,52).

Another approach for the enhanced intracellular delivery of ODN is by receptor mediated endocytosis. Recent examples include attachment of IL-1ß to ODN and subsequent targeted delivery to cells expressing the IL-1ß receptor on the surface (53), by way of the transferrin receptor (54,55) and by way of biotin or folate receptors (56,57). Utilizing pH sensitive immunoliposomes, a plasmid encoding the thymidine kinase (TK) gene was successfully delivered to TK$^-$ mouse L cells in tissue culture (58). Antibody targeted liposomes were also used for the intracellular delivery of ODN targeted against VSV. These liposome encapsulated oligomers were active in amounts of 10- to 100-fold lower than unencapsulated oligomer sequences (59).

The mechanism of oligonucleotide uptake and intracellular distribution has also been addressed. Conjugation of ODN to polylysine was found to greatly enhance the uptake. The internalization follows a classical endocytic pathway (60). When fluorescently labeled ODNs were microinjected into cells, however, the fluorescent label rapidly accumulated in the nucleus. This accumulation was found for diester, thioate and methylphosphonate ODNs. Nuclear accumulation seemed to be an energy-independent pathway (61). On the other hand, it was shown by way of cell fractionation that, on incubation of cells with ODN thioates, the ODNs are distributed into the cytoplasm (major), the nucleus (minor) and membranes (minor). From these results, together with the biological activity of the ODN, it was concluded that the lack of activity is not due to the ODNs inability to reach the target, but is rather dependent on the sequence (62). The subject of cellular permeation remains clouded by conflicting data. While fluorescence and radiolabelled studies clearly show enhanced cellular uptake of ODN conjugates, the subcellular localization of ODNs (i.e. are they for the most part sequestered and not available for target hybridization) remains unclear.

Structure Modifications - The simplest modification of the ionic phosphate backbone is the conversion to a phosphate triester. Such phosphate methyl esters had previously been prepared and claimed to show activity against HIV (63), but this result has since been retracted (64). Such methyl esters can also be prepared by a slightly different procedure (65). Duplexes formed between ODN diesters and ODN methyl phosphonates of defined stereochemistry show an enhanced thermal melting temperature (Tm) relative to their racemic counterparts (66). This observation makes the synthesis of stereoregular methyl phosphonate ODNs an attractive goal, and efforts to this end have been reported (67,68). Boranophosphates have appeared as synthetically accessible phosphate replacements which preserve the negative charge (69). Recent efforts have focused on the synthesis of neutral phosphate isosteres which lack a chiral center. Such backbone modifications synthesized as dimers include replacement of the phosphate by a methylene (70), sulfamate (71), sulfides, sulfoxides and sulfones (Figure 3) (72).

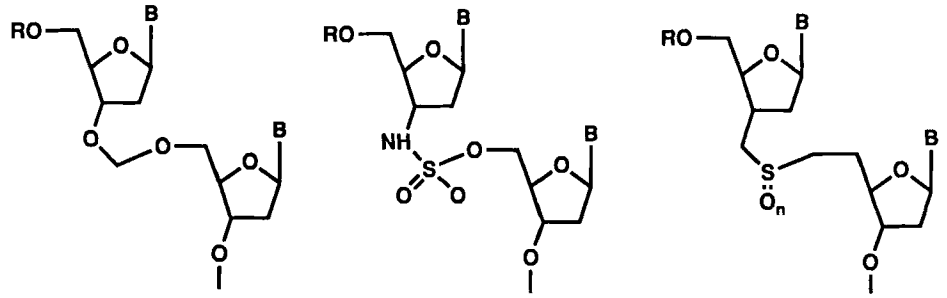


Figure 3. Formacetal, sulfamate, sulfoxide and sulfone internucleotide linkages.

One way to improve the biological potency of antisense ODNs is attachment to a reactive functional group. Thus, N-mustards as alkylating compounds (73), porphyrins (74,75) and psoralens as photochemical activatable agents (76,77) and quinones as inducible alkylating agents (78) have been investigated in this area.

The furanose oxygen has been replaced by a methylene to obtain carbocyclic ODNs which might have desirable properties (79,80). Other developments include the synthesis of enantiomeric DNA in which the natural (R)-ribose is replaced by the (L)-ribose (81). Such enantiomeric DNA was found to hybridize to complementary RNA but not DNA.

Cell-free and Cell-based Assays - In studies measuring inhibition of translation in cell-free extracts of trypanosomes, protein synthesis inhibition correlated with the affinity of the antisense ODN to its target RNA (82). Cell-based antisense effects reported include inhibition of neurite polarity growth in primary cerebellar neurons with an ODN complementary to the 5'-sequence of the tau gene (83). A diester ODN targeted against IL-1α was found to prevent senescence and extended the proliferative lifespan of human endothelial cells (84). The inhibition of c-Ha-ras p21 expression in NIH3T3 cells is target dependent, with an ODN pentadecamer targeted against the cap region showing greater activity than one

targeted against the 5'-untranslated region (85). A striking specificity of ODN antisense compounds was evident from targeting the first five codons of myogenin which blocks the stimulation of terminal myogenic differentiation by IGF-1, but none of the other processes stimulated by IGF-1 were affected (86). Also, an ODN targeted against a subunit of protein kinase RIIβ mRNA inhibited a cAMP-induced differentiation without affecting the effects of phorbol ester (87). An ODN directed against a c-myc intron sequence, responsible for a high proportion of Burkitt lymphomas, inhibited the proliferation of a lymphoma cell line containing these transcripts, but not the cell line containing the normal transcript RNA (88). A similar specificity was evident in the antisense inhibition of N-myc expression which resulted in decreased cell growth without reduction of c-myc protein levels (89). Both ODN diesters and thioates were compared in the inhibition of BCL2 protooncogene expression in leukemic cells. ODN thioates were more potent than diesters and can be potent inhibitors of leukemic cell growth and survival (90,91). Also, ODNs were found to be inhibitors of influenza virus replication (92), where thioates were more effective than diesters. Some unspecific effects were noted, namely the biological effect was also observed with mismatched ODN. Similar sequence unspecific antiviral effects of thioate ODNs were observed in a herpes simplex virus assay (93). ODN thioates cause a number of aberrations in cellular metabolism and histology on microinjection into Xenopus oocytes. The results suggest that some of the earlier reported antisense results might be partly due to these unspecific effects (94). Unspecific toxicity of microinjected ODN thioates in oocytes has also been noted (95).

To date, reported antisense RNA effects in tissue culture have required micromolar concentrations of ODN. These rather high concentrations can easily lead to non-sequence specific effects. Consequently, each antisense inhibition experiment must be repeated with several control sequences to verify the sequence-specific nature of the observed effect. From the standpoint of drug development, potency of the antisense ODNs will have to be improved in order to make this approach practical.

In Vivo Effects, Efficacy and Pharmacokinetics - ODN stability in subcellular extracts, culture media and cerebrospinal fluid was studied and showed that these ODNs were degraded slowly, with half lifes ranging from 7 to 19 hours (96). When ODN methylphosphonates were injected iv into mice, the distribution profile showed that the oligomer was rapidly distributed into all tissues and the radiolabeled ODN was excreted to appproximately 70% after 60-120 min in the urine (97).

SUMMARY

Targeting genomic DNA or mRNA in a sequence specific manner is a promising approach to very selective potential therapeutics. Targeting duplex DNA by triple helix formation is limited to polypurine-polypyrimidine sequences and it has been shown for *in vitro* cell free assay systems, but there is a paucity of biological effects in tissue culture assays. The targeting of mRNA with oligonucleotides through Watson-Crick base pairing has been employed in a number of *in vitro* tissue culture systems and results in sequence specific biological effects, although sequence unspecific effects have been noted in some cases. The concentration of oligonucleotides required is relatively high and efficient permeation of the oligomer into cells seems to be the major hurdle which has to be overcome to make this approach commercially feasible.

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SECTION VII. TRENDS AND PERSPECTIVES

Editor: James A. Bristol
Parke-Davis Pharmaceutical Research Division
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Chapter 31. To Market, To Market - 1990

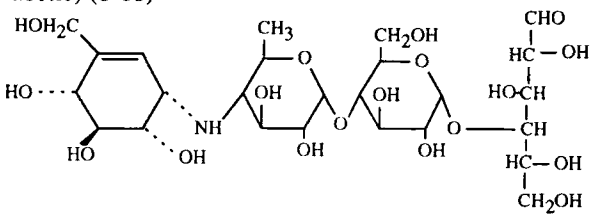
June D. Strupczewski, Daniel B. Ellis, and Richard C. Allen
Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876

The new chemical entities (NCEs) for human therapeutic use introduced into the world marketplace for the first time during 1990 totaled 37, up slightly in comparison with 33 in 1989 (1), but still somewhat under the 52 in 1988 (2) and 67 in 1987 (3).

Japan, as in recent years, held the lead with 11 NCE introductions, followed by the United Kingdom and Spain, with 5 and 4 entries, respectively. Over 40% of the new NCEs launched in 1990 originated in two countries: 9 in Japan and 7 in the United States; Germany, the United Kingdom, Spain, and Switzerland each had four. The cardiovasculars (including, two new ACE inhibitors and three new calcium channel blockers) continued to outnumber other categories during 1990, followed by agents for the treatment of CNS disorders, bronchopulmonary agents, and antiinfectives. NCEs of special interest include the first alpha-glucosidase inhibitor, acarbose, an adjuvant in the treatment of diabetes; the first selective $5HT_3$ receptor antagonist, ondansetron hydrochloride, for use in the management of nausea induced by cancer chemotherapy; and two new long acting bronchodilators, bambuterol and salmeterol hydroxynaphthoate.

During 1990, 19 NCE therapeutic agents were approved in the United States (4,5,6). From these, as well as those approved in previous years, a total of 23 reached the marketplace, including eight designated by the FDA as having a significant therapeutic gain, the highest number of such agents since this classification system was initiated. Two of the 23, nafarelin acetate and pegademase bovine, represent first worldwide introductions. By way of comparison, 25 NCEs were introduced into the UK market for the first time in 1990 (7).

Acarbose (antidiabetic) (8-10)



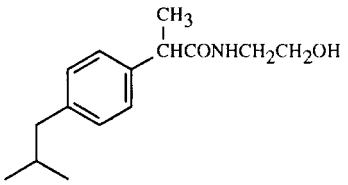
Country of Origin:	**Germany**	Introduced by:	**Bayer AG**
Originator:	**Bayer AG**	Trade Name:	**Glucobay**
First Introduction:	**Germany**	CAS Registry No.:	**56180-94-0**

Acarbose, a complex oligosaccharide isolated from Actinoplanes, is reportedly useful as an adjuvant therapy in diabetes. By inhibiting alpha-glucosidase, acarbose delays carbohydrate metabolism in the gastrointestinal tract and modulates changes in food induced blood sugar levels.

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Aminoprofen (topical antiinflammatory) (11, 12)

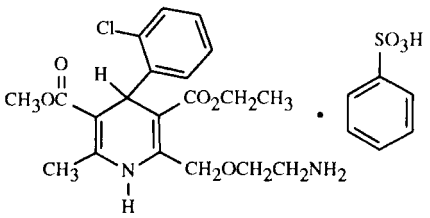
Country of Origin: **Spain**
Originator: **Aldo-Union**
First Introduction: **Spain**
Introduced by: **Aldo-Union**
Trade Name: **Aldospray Analgesico**
CAS Registry No.: **82821- 47- 4**



Aminoprofen, an amide of ibuprofen, is a topical anti-arthritic with analgesic properties. It is comparable to methyl salicylate in the carrageenan-induced paw edema and croton oil ear edema models, and is reported to be a superior analgesic.

Amlodipine Besylate (antihypertensive) (13-15)

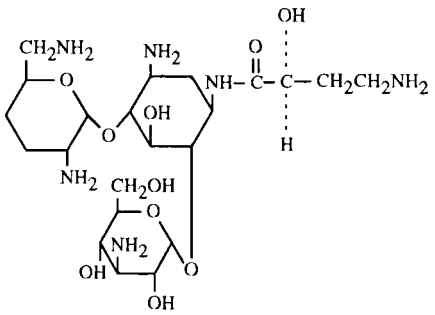
Country of Origin: **U. S. A.**
Originator: **Pfizer**
First Introduction: **Ireland**
Introduced by: **Pfizer**
Trade Name: **Istin**
CAS Registry No.: **111470-99-6**



Amlodipine besylate is a new, once-daily, dihydropyridine calcium antagonist useful in the treatment of hypertension and angina.

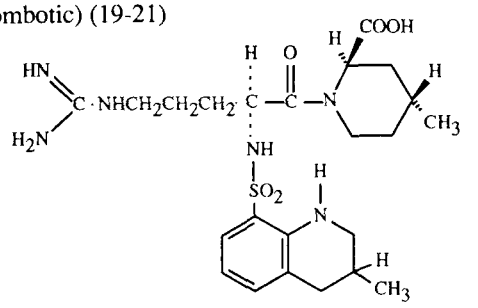
Arbekacin (antibiotic) (16-18)

Country of Origin: **Japan**
Originator: **Inst. Microbial Chemistry**
First Introduction: **Japan**
Introduced by: **Meiji Seika**
Trade Name: **Habekacin**
CAS Registry No.: **51025-85-5**



Arbekacin is a semi-synthetic derivative of dibekacin useful in the treatment of bacterial infections. This aminoglycoside is active against a broad spectrum of bacteria, including some of the gentamycin-, kanamycin-, and tobramycin-resistant pathogens. Compared to amikacin and dibekacin, ototoxicity is reportedly milder.

Argatroban (antithrombotic) (19-21)

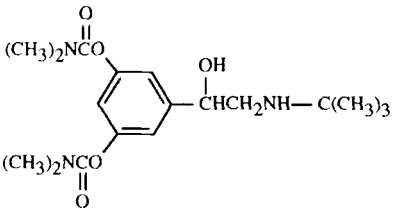


Country of Origin:	**Japan**	Introduced by:	**Mitsubishi Kasei; Daiichi**
Originator:	**Mitsubishi Kasei; Daiichi**	Trade Name:	**Slonnon; Novastan**
First Introduction:	**Japan**	CAS Registry No.:	**74863-84-6**

Argatroban is a new synthetic antithrombotic agent useful in maintenance anticoagulation therapy. In patients on hemodialysis, argatroban is superior to heparin, generating a more stable antithrombin effect. Other potential uses include progressing ischemic stroke and disseminated intravascular coagulation.

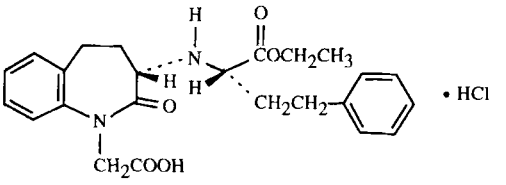
Bambuterol (bronchodilator) (22-24)

Country of Origin:	**Sweden**
Originator:	**Astra**
First Introduction:	**Sweden**
Introduced by:	**Draco (Astra)**
Trade Name:	**Bambec**
CAS Registry No.:	**81732-65-2**



Bambuterol is a new once-daily, oral bronchodilator especially useful in the chronic treatment of nocturnal asthma. It is a prodrug slowly converted by hydrolysis in the lung tissue to terbutaline, generating long-acting bronchodilation with comparable side effects.

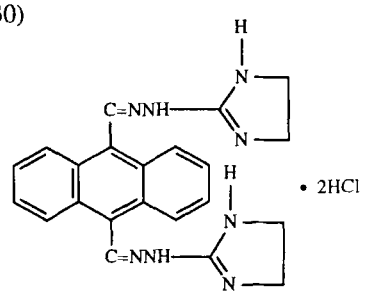
Benazepril Hydrochloride (antihypertensive) (25-27)



Country of Origin:	**Switzerland**	Introduced by:	**Ciba-Geigy**
Originator:	**Ciba-Geigy**	Trade Name:	**Cibacen**
First Introduction:	**Denmark**	CAS Registry No.:	**86541-74-4**

Benazepril hydrochloride, a prodrug of benazeprilat, is a long-acting angiotension-converting enzyme (ACE) inhibitor useful in the treatment of essential hypertension. In healthy humans, it was well tolerated and showed no pharmacokinetic interactions with furosemide, hydrochlorothiazide, chlorthalidone, digoxin, cimetidine, atenolol, or naproxen. Benazepril hydrochloride is under investigation as a cardiostimulant.

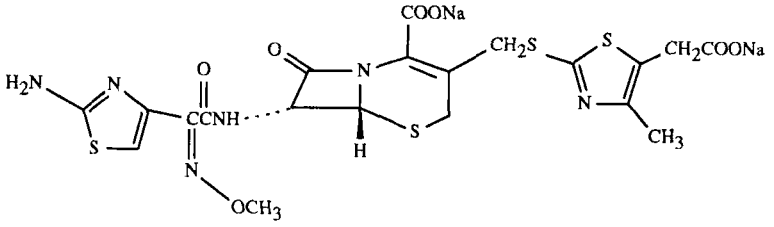
Bisantrene Hydrochloride (antineoplastic) (28-30)



Country of Origin: **U.S.A.**
Originator: **Lederle**
First Introduction: **France**
Introduced by: **Lederle**
Trade Name: **Zantrene**
CAS Registry No.: **71439-68-4**

Bisantrene hydrochloride is a cytostatic useful in the treatment of adult acute, non-lymphocytic leukemia. It is reportedly active against lymphoma, myeloma, bladder, lung and advanced breast cancer, but not small-cell lung cancer, renal cancer, melanoma or refractory adult acute leukemia.

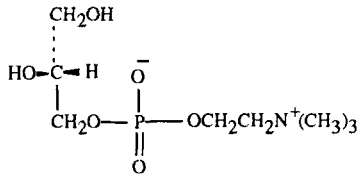
Cefodizime Sodium (antibiotic) (31-33)



Country of Origin: **Germany**
Originator: **Hoechst AG**
First Introduction: **Japan**
Introduced by: **Hoechst; Taiho**
Trade Name: **Neucef; Kenicef**
CAS Registry No.: **86329-79-5**

Cefodizime sodium is a parenteral, long-acting, broad-spectrum cephalosporin useful in the treatment of bacterial infections. It is efficacious against pathogens resistant to penicillins and older cephalosporins. An indication under study is its reported immunostimulatory activity.

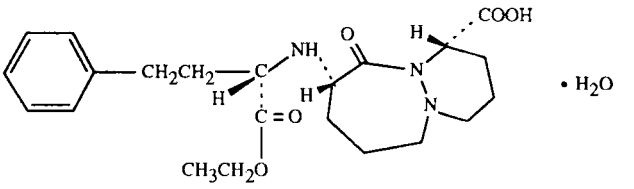
Choline Alfoscerate (nootropic) (34, 35)



Country of Origin: **Italy**
Originator: **Sandoz; Italfarmaco**
First Introduction: **Italy**
Introduced by: **LPB**
Trade Name: **Delecit**
CAS Registry No.: **28319-77-9**

Choline alfoscerate is a nootropic reportedly effective in the treatment of age-associated memory impairment. In man, it decreased and prevented scopolamine-induced amnesia. In vitro studies suggest that choline alfoscerate acts indirectly on cholinergic transmission by elevating the synthesis of acetylcholine.

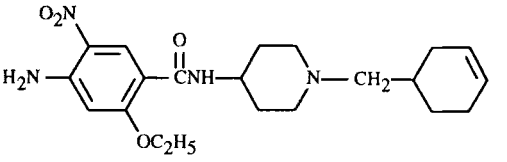
Cilazapril (antihypertensive) (36-38)



Country of Origin:	**Switzerland**	Introduced by:	**Hoffmann-LaRoche**
Originator:	**Hoffmann-LaRoche**	Trade Name:	**Inhibace**
First Introduction:	**Switzerland**	CAS Registry No.:	**92077-78-6**

Cilazapril is a new ACE inhibitor useful in the management of mild to moderate hypertension. Orally administered cilazapril is a prodrug which is de-esterified to its active diacid metabolite cilazprilat. Compared with enalapril and captopril, cilazapril is more potent and longer lasting.

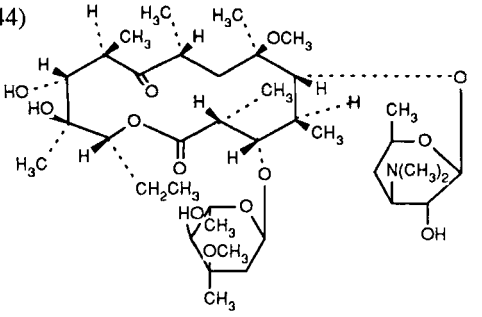
Cinitapride (gastroprokinetic) (39-41)



Country of Origin:	**Spain**	Introduced by:	**Almirall**
Originator:	**Almirall**	Trade Name:	**Cidine**
First Introduction:	**Spain**	CAS Registry No.:	**66564-14-5**

Cinitapride is a substituted benzamide dopamine receptor antagonist effective in the treatment of gastroesophageal reflux and a variety of gastrointestinal motility disorders. It generates strong procholinergic activity in the gastrointestinal tract by elevating the serotonergic transmission in the mysenteric plexus.

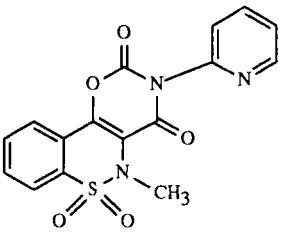
Clarithromycin (antibiotic) (42-44)



Country of Origin:	**Japan**
Originator:	**Taisho**
First Introduction:	**Ireland**
Introduced by:	**Abbott**
Trade Name:	**Klacid**
CAS Registry No.:	**81103-11-9**

Clarithromycin is an acid stable macrolide antibiotic indicated for use in the treatment of skin, urinary and respiratory tract infections. Compared to erythromycin, clarithromycin exhibits the same in vitro activity against conventional pathogens, but is better tolerated by generating less gastrointestinal problems.

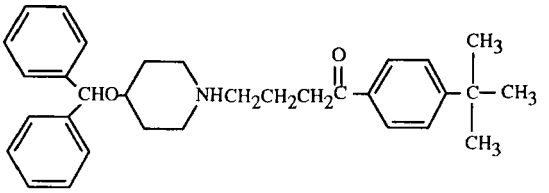
Droxicam (antiinflammatory) (45-47)



Country of Origin:	**Spain**
Originator:	**Esteve**
First Introduction:	**Spain**
Introduced by:	**Esteve**
Trade Name:	**Ombolan**
CAS Registry No.:	**90101-16-9**

Droxicam is an orally-active prodrug of piroxicam useful in the treatment of spinal osteoarthritis. Although it has a pharmacological profile comparable to piroxicam, droxicam's experimental ulcerogenic effect is ten times less. In animal studies droxicam is also reported to have analgesic and antipyretic activities, with no cardiovascular or respiratory effects.

Ebastine (antihistamine) (48-50)

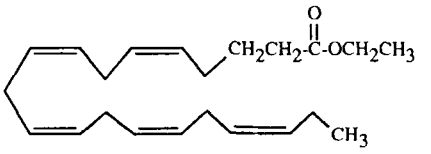


Country of Origin:	**Spain**	Introduced by:	**Almirall**
Originator:	**Almirall**	Trade Name:	**Ebastel**
First Introduction:	**Spain**	CAS Registry No.:	**90729-43-4**

Ebastine is a new once-daily histamine H_1-receptor antagonist with no sedative effects or autonomic impairment at therapeutic doses. It is reported to be effective in the treatment of hay fever, perennial rhinitis, and uticaria. Ebastine's antihistamine activity is attributed to its carboxylic acid metabolite carebastine.

Ethyl Icosapentate (antithrombotic) (51, 52)

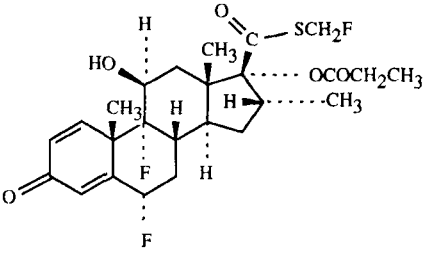
Country of Origin: **Japan**
Originator: **Nippon Suisan**
First Introduction: **Japan**
Introduced by: **Mochida**
Trade Name: **Epadel**
CAS Registry No.: **86227-47-6; 84494-70-2**



Ethyl icosapentate is an ester of a polyunsaturated acid derived from fish oil. It is reported to decrease platelet aggregation, reduce blood viscosity and increase erythrocyte deformability.

Fluticasone Propionate (antiinflammatory) (53)

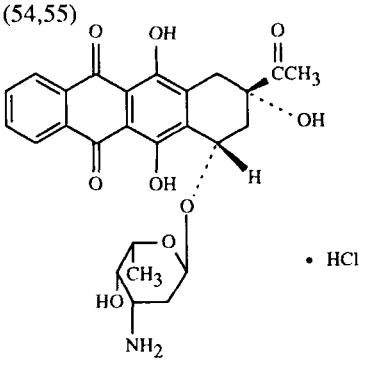
Country of Origin: **United Kingdom**
Originator: **Glaxo**
First Introduction: **United Kingdom**
Introduced by: **Glaxo**
Trade Name: **Flixonase**
CAS Registry No.: **80474-14-2**



Fluticasone propionate is a new glucocorticosteroid useful in the treatment of seasonal rhinitis and asthma. Compared to beclomethasone dipropionate, fluticasone propionate is reported to have a comparable side effect profile, but twice the activity in the treatment of asthma.

Idarubicin Hydrochloride (antineoplastic) (54,55)

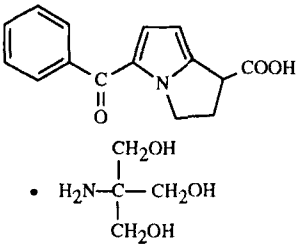
Country of Origin: **Italy**
Originator: **Erbamont**
First Introduction: **United Kingdom**
Introduced by: **Erbamont**
Trade Name: **Zavedos**
CAS Registry No.: **57852-57-0**



Idarubicin hydrochloride is a derivative of daunorubicin indicated for acute non-lymphocytic leukemia, acute lymphocytic leukemia, and acute myeloid leukemia. Compared with daunorubicin, idarubicin hydrochloride is less cardiotoxic, has milder side effects, is orally active and more potent in experimental leukemias. Idarubicin hydrochloride is also reportedly active in daunorubicin-resistant patients, breast cancer, Hodgkin's and non-Hodgkin's lymphoma.

Ketorolac Tromethamine (analgesic) (56-58)

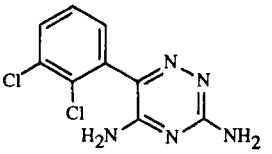
Country of Origin: **U.S.A.**
Originator: **Syntex**
First Introduction: **U.S.A.**
Introduced by: **Syntex; Roche**
Trade Name: **Toradol IM**
CAS Registry No.: **74103-07-4**



Ketorolac tromethamine is a nonsteroidal antiinflammatory agent that exhibits analgesic and antipyretic activity. The compound is effective in the management of moderate to severe postoperative pain. It is, however, the first of this type of agent to be administered parenterally as an analgesic and is specifically indicated for intramuscular injection. Ketorolac represents a useful alternative to the narcotic analgesics due to its lack of abuse potential.

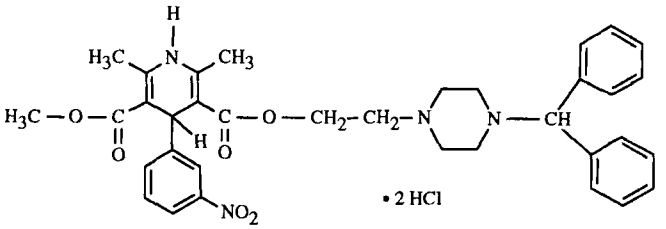
Lamotrigine (anticonvulsant) (59-61)

Country of Origin: **United Kingdom**
Originator: **Burroughs Wellcome**
First Introduction: **Ireland**
Introduced by: **Burroughs Wellcome**
Trade Name: **Lamictal**
CAS Registry No.: **84057-84-1**



Lamotrigine is a new triazine, glutamate inhibitor anticonvulsant that significantly reduces the incidence of refractory partial seizures. The drug is reported to produce fewer CNS side effects than diazepam or sodium phenytoin. It is also indicated as add-on therapy for the treatment of generalized seizures not satisfactorily controlled by other anti-epileptics.

Manidipine Hydrochloride (antihypertensive) (62-64)

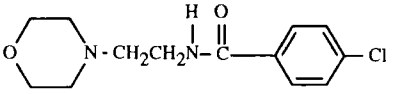


Country of Origin: **Japan**
Originator: **Takeda**
First Introduction: **Japan**
Introduced by: **Takeda**
Trade Name: **Calslot**
CAS Registry No.: **126229-12-7**

Manidipine hydrochloride is another second-generation dihydropyridine calcium antagonist introduced as an antihypertensive. In animal studies, manidipine hydrochloride dose-dependently decreased systolic pressure with potent and long-lasting effects. It has little cardiodepressant activity and its claimed to be useful in hypertensive patients without the fear of cardiac failure or severe bradycardia.

Moclobemide (antidepressant) (65, 66)

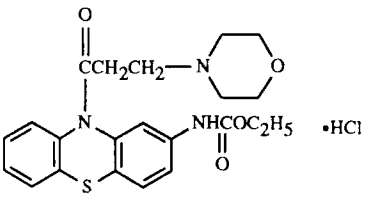
Country of Origin: **Switzerland**
Originator: **Hoffmann-LaRoche**
First Introduction: **Sweden**
Introduced by: **Hoffman-LaRoche**
Trade Name: **Aurorix**
CAS Registry No.: **71320-77-9**



Moclobemide is the first of a new generation of non-hydrazine, reversible MAO-A inhibitors useful in the treatment of depression. Moclobemide is a selective inhibitor of MAO-A, allowing tyramine to be metabolized by MAO-B. In controlled studies, moclobemide was clinically superior to desipramine and showed no cholinergic or cardiovascular side-effects. A metabolite is currently under investigation for treatment of Parkinson's disease.

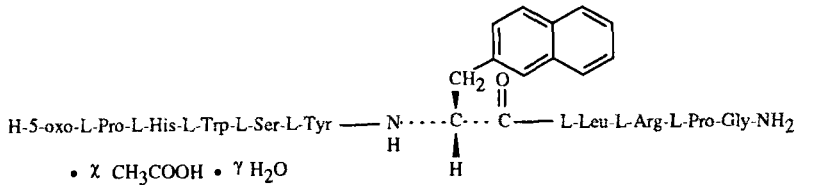
Moricizine Hydrochloride (antiarrhythmic) (67-69)

Country of Origin: **USSR**
Originator: **Acad. Med. Sci.**
First Introduction: **USSR**
Introduced by: **Alaine Pharm.**
Trade Name: **Ethmozine**
CAS Registry No.: **29560-58-8**



Moricizine hydrochloride is a phenothiazine derivative with antiarrhythmic activity developed by the Soviet Academy of Medical Sciences. It is licensed to Dupont-Sanko in a joint venture. Moricizine hydrochloride is the first drug licensed to a U.S. company by the Soviet Union and was introduced by Dupont in the U.S. in 1990 for specific use in life-threatening ventricular arrhythmias. This narrow indication reflects its proarrhythmic potential. The incidence of this adverse reaction, however, is lower than that of other antiarrhythmic agents.

Nafarelin Acetate (hormone) (70-72)

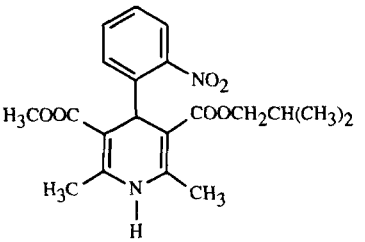


Country of Origin:	**USA**	Introduced by:	**Syntex**
Originator:	**Syntex**	Trade Name:	**Synarel**
First Introduction:	**USA**	CAS Registry No.:	**86220-42-0**

Nafarelin acetate is a synthetic decapeptide agonist analog of the natural gonadotropin-releasing hormone, differing in the amino acid at position 6 where D-naphthylalanine has been substituted for glycine. This substitution gives nafarelin greater potency and a substantially longer half-life than the endogenous hormone. Nafarelin acetate administered as a nasal spray, suppresses estrogen levels on repeated dosing and is indicated for the management of endometriosis. It also has orphan drug status for use in precocious puberty.

Nisoldipine (antihypertensive) (73-75)

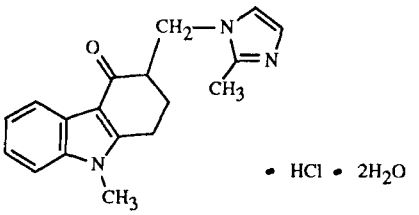
Country of Origin:	**Germany**
Originator:	**Bayer AG**
First Introduction:	**Japan**
Introduced by:	**Bayer**
Trade Name:	**Baymycard**
CAS Registry No.:	**63675-72-9**



Nisoldipine is another long-acting dihydropyridine calcium channel blocker effective in the treatment of hypertension and angina pectoris. It is reported to have little or no cardiodepressive activity and to be more effective than nifedipine in reducing blood pressure in hypertensives. Nisoldipine has also been shown to be effective in the management of congestive heart failure. Reduction in dosage is necessary in patients with hepatic, but not renal impairment.

Ondansetron Hydrochloride (antiemetic) (76-79)

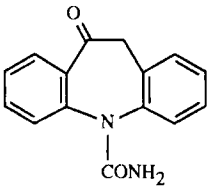
Country of Origin:	**United Kingdom**
Originator:	**Glaxo**
First Introduction:	**United Kingdom**
Introduced by:	**Glaxo**
Trade Name:	**Zophran**
CAS Registry No.:	**103639-04-9**



Ondansetron hydrochloride is a selective $5HT_3$-antagonist approved for the management of nausea and vomiting induced by cancer chemotherapy and radiotherapy. It is reported to have a superior side-effect profile compared with metoclopramide, representing an important advance in the treatment of nausea and vomiting associated with chemotherapy. Ondansetron hydrochloride is the first specific $5HT_3$-antagonist to reach the market. It is also under investigation for a number of other indications, including anxiety and schizophrenia.

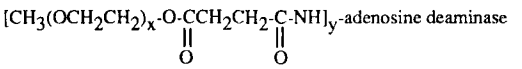
Oxcarbazepine (anticonvulsant) (80, 81)

Country of Origin:	**Switzerland**
Originator:	**Ciba-Geigy**
First Introduction:	**Denmark**
Introduced by:	**Ciba-Geigy**
Trade Name:	**Trileptal**
CAS Registry No.:	**28721-07-5**



Oxcarbazepine is a new antiepileptic carbamazepine derivative, reportedly better tolerated than carbamazepine. It appears to be most effective in partial epilepsy with complex seizures.

Pegademase Bovine (immunostimulant) (82, 83)



x ≈ 114 oxyethylene groups per PEG strand

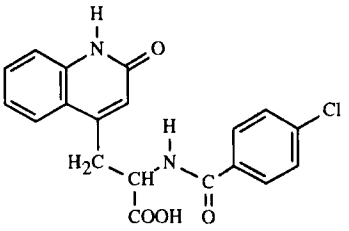
y ≈ 11-17 primary amino groups of lysine on to which succinyl PEG is attached

Country of Origin:	**USA**	Introduced by:	**Enzon**
Originator:	**Enzon**	Trade Name:	**Adagen**
First Introduction:	**USA**	CAS Registry No.:	**130167-68-9**

Pegademase bovine is an orphan drug for the treatment of severe combined immunodeficiency disease due to the lack of adenosine deaminase. It is specifically indicated for those patients who are not suitable for, or who have had unsuccessful bone marrow transplantation. Attachment of PEG residues to the native enzyme results in significant increase in circulating half-life and reduction in allergic potential.

Rebamipide (antiulcer) (84, 85)

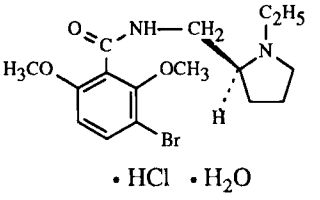
Country of Origin: **Japan**
Originator: **Otsuka**
First Introduction: **Japan**
Introduced by: **Otsuka**
Trade Name: **Mucosta**
CAS Registry No.: **111911-87-6**



Rebamipide is a quinolinone derivative introduced as a novel antiulcer agent. It acts as a local gastric mucosa prostaglandin inducer and is reported to be effective in both the healing and prevention of recurrence of gastric ulcer.

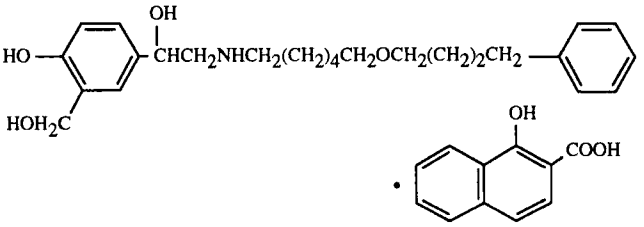
Remoxipride Hydrochloride (antipsychotic) (86)

Country of Origin: **Sweden**
Originator: **Astra**
First Introduction: **Denmark**
Introduced by: **Astra**
Trade Name: **Roxiam**
CAS Registry No.: **82935-42-0**



Remoxipride hydrochloride is a new selective dopamine D_2-antagonist for the treatment of schizophrenia and other psychoses where thought disorders are prominent symptoms. Efficacy appears to be similar to that of haloperidol, with reportedly significantly less extrapyramidal side-effects.

Salmeterol Hydroxynaphthoate (bronchodilator) (87-89)

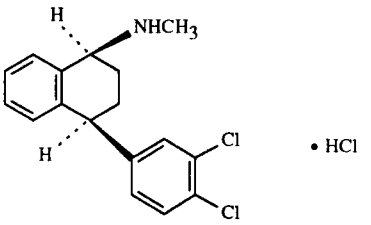


Country of Origin: **United Kingdom**
Originator: **Glaxo**
First Introduction: **United Kingdom**
Introduced by: **Glaxo**
Trade Name: **Serevent**
CAS Registry No.: **94749-08-3**

Salmeterol hydroxynaphthoate is a new long-acting β_2-selective adrenoceptor agonist introduced as a bronchodilator for the treatment of reversible airway obstruction in asthma and chronic bronchitis. It has bronchodilatory activity as intense and four times longer-lasting than equivalent doses of salbutamol. Salmeterol hydroxynaphthoate is reported to be the first bronchodilator with significant anti-inflammatory activity and hence could complement prophylactic corticosteroid therapy.

Sertraline Hydrochloride (antidepressant) (90-92)

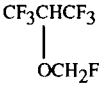
Country of Origin: **USA**
Originator: **Pfizer**
First Introduction: **United Kingdom**
Introduced by: **Pfizer**
Trade Name: **Lustral**
CAS Registry No.: **79559-97-0**



Sertraline hydrochloride is a selective serotonin reuptake inhibitor, similar mechanistically to fluoxetine, introduced initially for the maintenance and long-term treatment of depression. It is reportedly non-sedating and compared to fluoxetine has a shorter duration of action and lack of CNS activating effects such as nervousness and anxiety. Sertraline hydrochloride is currently undergoing investigation for the treatment of obesity and obsessive-compulsive disorders.

Sevoflurane (anesthetic) (93, 94)

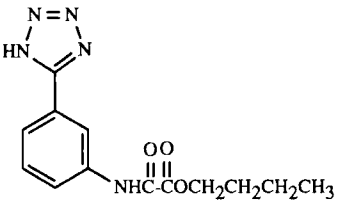
Country of Origin: **USA**
Originator: **Baxter**
First Introduction: **Japan**
Introduced by: **Maruishi**
Trade Name: **Sevofrane**
CAS Registry No.: **28523-86-6**



Sevoflurane is a rapidly acting, potent inhalation anesthetic with rapid uptake and and elimination. While somewhat less potent than halothane, sevoflurane does not induce arrhythmias and reportedly has no effect on renal function.

Tazanolast (antiallergic) (95)

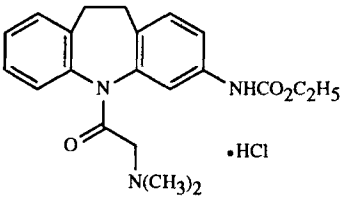
Country of Origin: **Japan**
Originator: **Wakamoto**
First Introduction: **Japan**
Introduced by: **Wakamoto; Torii**
Trade Name: **Tazalest; Tazanol**
CAS Registry No.: **82989-25-1**



Tazanolast is an orally active antiallergic agent useful in the treatment of bronchial asthma and nasal allergy. Mechanistically it appears to inhibit IgG and IgE-mediated antigen-antibody reactions.

Tiracizine Hydrochloride (antiarrhythmic) (96, 97)

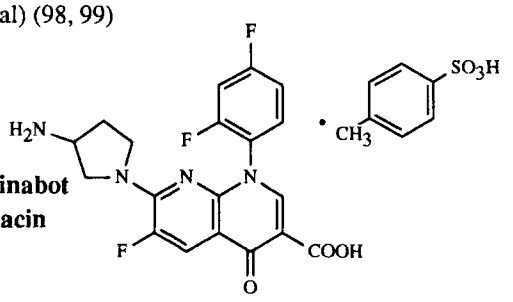
Country of Origin: **Germany**
Originator: **GERMED Dresden**
First Introduction: **USSR; Germany**
Introduced by: **GERMED Dresden**
Trade Name: **Bonnecor**
CAS Registry No.: **78816-67-8**



Tiracizine hydrochloride is a antiarrhythmic reported to reduce excitability of cardiac cells via blockade of fast sodium channels.

Tosufloxacin Tosylate (antibacterial) (98, 99)

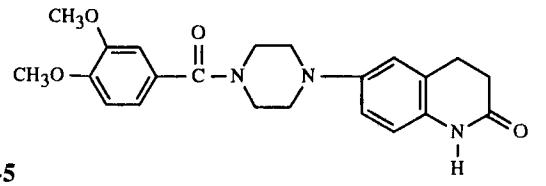
Country of Origin: **Japan**
Originator: **Toyama**
First Introduction: **Japan**
Introduced by: **Toyama; Dainabot**
Trade Name: **Ozex; Tosuxacin**
CAS Registry No.: **115964-29-9**



Tosufloxacin tosylate is a new fluoroquinolone antibacterial structurally related to temafloxacin. Mechanistically, it is a microbial DNA topoisomerase inhibitor, useful against beta-lactamase producing organisms. Tosufloxacin tosylate is reported to be particularly effective against Salmonella enteritidis.

Vesnarinone (cardiostimulant) (100, 101)

Country of Origin: **Japan**
Originator: **Otsuka**
First Introduction: **Japan**
Introduced by: **Otsuka**
Trade Name: **Arkin -Z**
CAS Registry No.: **81840-15-5**



Vesnarinone is a new orally-active positive inotropic agent introduced for the treatment of congestive heart failure. It exerts a positive inotropic effect on the heart and has a peripheral vasodilatory effect. Vesnarinone is claimed not to affect heart rate or aggravate arrhythmia.

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Chapter 32. Perspectives on Human Gene Therapy

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Introduction - Despite many advances in understanding eukaryotic gene expression, a major obstacle to the treatment of human disease remains the inability to express genes stably at specific sites in vivo. Recent progress in the regulation of eukaryotic transcription and viral gene expression has made it possible to address fundamental questions relevant both to basic biology and to human disease. At the same time, gene transfer technology has also provided access to a variety of recombinant gene products that can be applied to clinical medicine for diagnostic and therapeutic purposes. In this perspective, recent developments and future potential of this technology and its application to human gene therapy have been reviewed. It is now clear that gene therapy will prove therapeutic in a variety of diseases. Progress has been made in a variety of systems. Potential host cells may be derived from the bone marrow, liver, lymphoid system, central nervous system, and lung, and several recent reviews have described progress in these cell types (1-3). The recent identification and correction of the genetic defect in cystic fibrosis (4-8) has proceeded rapidly. Newer approaches to the treatment of malignancy (9), AIDS (10), and hyperlipidemia (11) are also progressing. Another recent development has been the ability to deliver recombinant genes to cells of the vessel wall. This technology provides a novel approach to treat cardiovascular disease, malignancy, and AIDS, and is a potential method for drug delivery. In this review, this new area of gene therapy is reviewed as a representative example of the potential for gene therapy to impact upon human disease.

Definition of Terms - Over the past decade, gene therapy has emerged as a potential treatment modality for inherited disorders. This technology involves the insertion of normal genes into somatic cells to correct an inherited or acquired disorder through the synthesis of missing or defective gene products in vivo. A variety of approaches to human gene transfer are currently under experimental investigation. Various mechanical, chemical, and viral methods have been employed to introduce recombinant genes into host cells of different organ systems. In principle, human disease can arise from defects in the coding sequence of a gene or from aberrant regulation of gene expression. Significant progress has been made in the identification of mutations of gene products which result in human disease. So initial efforts towards human gene therapy have focused on providing the correct gene product.

To correct a missing or defective gene, three methods can be used: gene replacement, gene correction, and gene augmentation. Gene replacement requires the insertion of a normal, functional gene and the specific removal of the mutant gene sequence from the host genome. Although the synthesis of normal gene products has been achieved in many instances, for example, adenosine deaminase deficiency (12), removal of the defective gene is currently impractical for human gene therapy, although several promising approaches are under development in the laboratory.

The aim of gene correction is to alter the defective portion of a mutant gene within the genome to render the gene functional. Although gene correction is technically possible, it is also difficult to achieve. Recently, genetic targeting of foreign sequences leading to specific sequence modification has been accomplished in several mammalian systems in tissue culture and in transgenic animals (13-15), but is not yet likely to be used for human gene therapy, since the efficiency of homologous recombination is low in higher eukaryotic cells (13-15).

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Gene augmentation is a more feasible technique for modifying the expression of mutant genes in defective cells, by introducing normal genetic sequences into the host genome and leaving the host genes unaltered (2). This technique restores the genetic function in these cells by the introduction of the correct gene randomly in the host genome without removal of the nonfunctional mutant gene. Although mutational events could arise from the integration of foreign sequences at ectopic sites in the genome, this complication has not yet proven problematic in practice. For this reason, gene augmentation has received considerable attention for gene therapy.

Gene Transfer Vectors - To successfully deliver DNA to host cells, a gene transfer system must meet several requirements, including (a) high efficiency of transmission; (b) maintenance of foreign DNA in a stable form, either integrated in the host genome or as an extrachromosomal element; (c) expression in the relevant tissue; (d) appropriate and regulated expression in the target tissue; and (e) adequate safety at the time of transfer and during the life of the host.

A variety of techniques have been used in the laboratory to introduce genes into cells in tissue culture, including precipitation with calcium phosphate, polycations or polymers to complex with DNA, encapsidation of DNA into liposomes, or delivery with microspheres (16,17). A general characteristic of most of these transfection methods is the integration of multiple repeated copies of the foreign gene into the genome in a relatively stable form. These methods are investigational and have not been used in clinical protocols.

Viral vectors can also provide an efficient delivery system for the introduction genes into host cells. Retroviruses and other vectors are thought to integrate almost entirely into random sites in the host cell genome. As with other delivery systems, random integration could lead to insertional mutagenesis through the interruption of cellular genes or the modulation of cellular gene expression by insertion into regulatory sequences. Another potential problem with viral vectors is the possible generation of wild-type virus through recombination between the transfected vector plasmid and endogenous viruses. The possibility of productive recombination can often be minimized. For example, retroviral vectors can be packaged in helper cell lines that express the gag, pol, and env genes which have been produced from separate plasmids with independent selectable markers which would require multiple recombination events to generate wild-type virus (18).

The ability to introduce recombinant genes directly into the arterial wall provides an opportunity to perform gene therapy by the direct introduction of DNA. Given the potential hazards of random integration and expression of specific genes in inappropriate cells, it is important that the expression of such genes be controlled. Several approaches can be used to regulate the expression of such genes in vivo. First, suicide vectors which contain selectable markers will allow elimination of cells containing the recombinant gene in the event of cell transformation. Such a vector using the herpesvirus thymidine kinase gene has recently been synthesized (19). When this gene is introduced into tumor cells and implanted into mice, large tumors are eliminated completely by providing the drug, ganciclovir, which is phosphorylated selectively by this herpesvirus gene to a form which inhibits DNA replication. It has no effect on tumor cells which lack the recombinant thymidine kinase gene.

A second approach is to achieve specificity of expression through the use of tissue specific enhancer elements in expression vectors. For these purposes, a variety of cell specific promoters or enhancers have been defined and will be useful in directing recombinant gene expression to appropriate cells in vivo. Finally, the regulated expression of such genes is important. Using knowledge of cellular transcription factors and regulatory elements, it should soon be possible to design inducible promoters which provide for regulated expression of recombinant genes. Such promoters could contain different combinations of inducible elements such as the metallothionein, AP1, or kB elements, in combination with various cell specific promoters to reduce basal levels of transcription in transduced cells.

Gene Transfer into the Vasculature - Expression of recombinant genes in the vessel wall may help to achieve several goals. It will allow analysis of the activity of gene products in vivo; many have already been described in vitro. Function in the vessel can be determined, as well as effects when delivered into specific microcirculations. Another long-term goal is to achieve drug delivery using endothelial cells to secrete gene products continuously in vivo.

Site-specific gene expression in vivo must be addressed at two levels: 1) the site in the vasculature where a target gene is expressed, and 2) the cell type within the vessel wall where the recombinant gene will be synthesized. For example, if a gene is expressed in an endothelial cell, it could be secreted into the circulation or released locally and exert effects on adjacent endothelial and vascular smooth muscle cells in the intimal and proximal medial layer of the artery. If gene expression is limited to the vascular smooth muscle cell, a more restricted effect on the proliferation of surrounding vascular smooth muscle cells might be seen.

Cell-Mediated Gene Transfer - Two approaches to the delivery of genes into vessels have been undertaken. First, model systems have been established to introduce recombinant genes into specific cells from the vasculature in vitro. Once expression has been confirmed, these cells are reintroduced into the arterial wall (20). Endothelial cells can be modified in tissue culture using retroviral vectors or by lipofection and used as cell vehicles to introduce recombinant genes in vivo (21). Similarly, other cell types from the vessel, for example, vascular smooth muscle cells can be transduced and reintroduced into the arterial wall. The approach to reintroduce these cells makes use of a double-balloon catheter which is inserted into the artery. Both balloons are inflated simultaneously, isolating a central space which is protected from the circulation. After normal endothelium is removed, a central instillation port allows introduction of cells. By electron microscopy, mechanical denudation causes exposure of a collagenous basement membrane structure. Large crevices appear in the denuded vascular wall, allowing penetration of transduced cells into the wall. Cells applied through the instillation port of the catheter attach to this surface. In arteries with transduced endothelial cells, a layer of cells which contain the recombinant gene can be visualized (20). Vascular smooth muscle cells have also now been transduced with recombinant genes and inserted into vessels (21). In this case, cells can be visualized in layers below the internal elastic lamina as well as the neointima. Recombinant genes can therefore be introduced into cell types, both endothelial cells and vascular smooth muscle cells, and transplanted where they demonstrate stable expression in vessels. This technology has also been used to deliver recombinant gene products to prosthetic devices. Endothelial cells modified with recombinant genes in vitro (22) can be introduced into prosthetic graft or stents (23,24). Expression of thrombolytic or anti-coagulant genes in these cells could improve the performance of such devices, for example, by minimizing thrombotic complications.

Direct Gene Transfer In Vivo - From a practical and a clinical perspective, several problems remain in adapting cell-mediated gene transfer to the treatment of human disease. To introduce a recombinant gene into the vessel, an autologous cell line must be derived and transduced prior to its introduction into patients. Both from an experimental and practical point of view, the ability to directly introduce a recombinant gene represents a major technical advance, eliminating the time and effort of preparing cells in advance of the procedure. Using the same catheter system described above, direct gene transfer has been accomplished, modifying the procedure by introducing either a retrovirus or a DNA liposome complex through the instillation port onto the arterial wall (25). By direct infection with retroviral vectors, high levels of expression can be seen for over five months, although a diminution is observed with time. Microscopic analysis of these vessels shows that recombinant gene expression also occurs in several cell types including endothelial cells, vascular smooth muscle cells, and in the adventitia (25). This technique therefore allows direct delivery and provides the advantage, in the case of human diseases, that no prior preparation of cells or tissue is required. A potential disadvantage is the loss of control over the cell types into which the gene will be introduced; however, other refinements, for example, the use of tissue specific enhancers, may overcome this problem.

Other methods for the direct introduction of recombinant genes have also been described. In skeletal muscle, for example, injection of DNA into tissue results in uptake and recombinant gene expression (26). This technology may have applications to the treatment of muscle diseases and may also be useful for immunization using recombinant genes. This approach has recently proven successful as a means to introduce recombinant genes into the myocardium and will provide another approach to gene therapy for cardiovascular disease (27-29).

Biologic Effects of Gene Transfer In Vivo - The principle that recombinant genes can be introduced directly in vivo has been demonstrated (25,26). More recently, it has been possible to document a biologic response to a recombinant gene on the vessel wall. Initially, this has been achieved

using the transplantation response, and demonstrating an immune response to a foreign transplantation antigen expressed in vivo. This response was specific, both for transplantation antigen and for the localized region of the vessel where the gene was introduced. These studies show that a specific response to the introduction of a particular gene product can be achieved in vivo. This specific system will provide a model for the study of vasculitis. It may also have applications to human disease in instances where a significant degree of local immunity may be desirable.

Future Directions - Another goal of this approach to gene therapy is its application to systemic drug delivery. In any vessel, a limited number of endothelial cells, 104 - 105 cm2, are available; however, it would be necessary to transduce a much larger number of cells to make sufficient gene product to detect it circulating in the blood. This goal could be accomplished, for example, by transducing cells in the capillary circulation. Several experiments have indicated promise in this regard. The question remains whether sufficient expression can be achieved to release detectable amounts of recombinant gene product into the circulation and whether it can be sustained. In cases where a recombinant gene is synthesized to replace a missing product, it is possible that such immunosuppression may be required to overcome this problem.

Another major concern of gene therapy in vivo is the ability to exert control in the event of unforeseen complications. Three approaches can be taken to terminate or regulate gene expression. First, the development of tissue specific promoters will allow specific expression to appropriate cell types. Second, the generation of inducible promoters will allow selective and regulated expression in vivo. Third, a suicide retroviral vector, as described above, will allow cells to be eliminated in the event of an undesirable phenotype.

Conclusions - *In the field of vascular gene transfer, recombinant gene expression has been* achieved in both endothelial and smooth muscle cells in vivo. Recombinant genes can also be introduced directly into localized vessel segments, and induce changes in the biologic function of vessels. Regulatable promoters and suicide retroviral vectors are also now becoming a reality, and some recombinant genes are beginning to be expressed and secreted into the circulation. The many advances in endothelial cell biology, thrombosis, hemostasis, retrovirology, and eukaryotic gene expression have provided the basis for these promising new developments. These and other systems will allow new approaches to understanding the biology and treatment of human diseases. The recent progress in the technology of gene transfer in vivo has raised the prospect that it will be used soon for the treatment of a variety of diseases. Recombinant genes have already been introduced within lymphocytes to provide markers to follow cells in vivo (9), and the introduction of potentially therapeutic genes has begun. Although initially intended for the treatment of inherited disorders, it has become increasingly clear that its major benefits will derive from the treatment of acquired diseases. The next major challenge is to identify recombinant genes which can serve therapeutic roles and to facilitate their development. Although several cell-mediated gene transfer protocols have been initiated, it is likely that human clinical trials using direct gene transfer will also become a reality in the foreseeable future. If the toxicity and complications of these protocols prove acceptable, gene therapy may soon fulfill its promise of bringing the power of molecular biology to the treatment of human disease.

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acarbose	antidiabetic	1990	26, 297
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
alminoprofen	analgesic	1983	19, 314
alpha-1 antitrypsin	protease inhibitor	1988	24, 297
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
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
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
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benazepril hydrochloride	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
betaxolol HCl	antihypertensive	1983	19, 315
bevantolol HCl	antihypertensive	1987	23, 328
bifemelane HCl	nootropic	1987	23, 329
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisantrene hydrochloride	antineoplastic	1990	26, 300
bisoprolol fumarate	antihypertensive	1986	22, 317
bopindolol	antihypertensive	1985	21, 324
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317

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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
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
camostat mesylate	antineoplastic	1985	21, 325
carboplatin	antibiotic	1986	22, 318
carumonam	antibiotic	1988	24, 298
cefbuperazone sodium	antibiotic	1985	21, 325
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefotetan disodium	antibiotic	1984	20, 317
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
cefpodoxime proxetil	antibiotic	1989	25, 310
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celiprolol HCl	antihypertensive	1983	19, 317
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
choline alfoscerate	nootropic	1990	26, 300
cibenzoline	antiarrhythmic	1985	21, 325
cicletanine	antihypertensive	1988	24, 299
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
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
diacerein	antirheumatic	1985	21, 326
dilevalol	antihypertensive	1989	25, 311

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dopexamine	cardiostimulant	1989	25, 312
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
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
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
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
fisalamine	intestinal antiinflammatory	1984	20, 318
flomoxef sodium	antibiotic	1988	24, 302
fluconazole	antifungal	1988	24, 303
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
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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
fotemustine	antineoplastic	1989	25, 313
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303

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gemeprost	abortifacient	1983	19, 319
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
goserelin	hormone	1987	23, 336
guanadrel sulfate	antihypertensive	1983	19, 319
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
imipenem/cilastatin	antibiotic	1985	21, 328
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indobufen	antithrombotic	1984	20, 319
interferon, gamma	antiinflammatory	1989	25, 314
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
lamotrigine	anticonvulsant	1990	26, 304
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
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
lomefloxacin	antibiotic	1989	25, 315
lonidamine	antineoplastic	1987	23, 337
loprazolam mesylate	hypnotic	1983	19, 321
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
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

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mexazolam	anxiolytic	1984	20, 321
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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
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
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
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
piketoprofen	topical antiinflammatory	1984	20, 322
pimaprofen	topical antiinflammatory	1984	20, 322
pinacidil	antihypertensive	1987	23, 340
pirarubicin	antineoplastic	1988	24, 309
piroxicam cinnamate	antiinflammatory	1988	24, 309

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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
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
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
ronafibrate	hypolipidemic	1986	22, 326
rosaprostol	antiulcer	1985	21, 332
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	*antiulcer*	1987	23, 342
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
schizophyllan	immunostimulant	1985	22, 326
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
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
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
tazanolast	antiallergic	1990	26, 309
teicoplanin	antibacterial	1988	24, 311
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terconazole	*antifungal*	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333

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tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
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
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
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

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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
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
propofol	ANESTHETIC	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
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
tiracizine hydrochloride		1990	26, 310
amlexanox	ANTIASTHMATIC	1987	23, 327
ibudilast		1989	25, 313
ciprofloxacin	ANTIBACTERIAL	1986	22, 318
enoxacin		1986	22, 320
norfloxacin		1983	19, 322
ofloxacin		1985	21, 331
pefloxacin mesylate		1985	21, 331
rifapentine		1988	24, 310
teicoplanin		1988	24, 311
tosufloxacin tosylate		1990	26, 310
arbekacin	ANTIBIOTIC	1990	26, 298
aspoxicillin		1987	23, 328
astromycin sulfate		1985	21, 324

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azithromycin		1988	24, 298
aztreonam		1984	20, 315
carboplatin		1986	22, 318
carumonam		1988	24, 298
cefbuperazone sodium		1985	21, 325
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
cefpimizole		1987	23, 330
cefpiramide sodium		1985	21, 325
cefpodoxime proxetil		1989	25, 310
ceftazidime		1983	19, 316
cefteram pivoxil		1987	23, 330
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
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
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
setiptiline		1989	25, 318
sertraline hydrochloride		1990	26, 309
tianeptine sodium		1983	19, 324

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toloxatone		1984	20, 324
acarbose	ANTIDIABETIC	1990	26, 297
tolrestat		1989	25, 319
ondansetron hydrochloride	ANTIEMETIC	1990	26, 306
fenticonazole nitrate	ANTIFUNGAL	1987	23, 334
fluconazole		1988	24, 303
itraconazole		1988	24, 305
naftifine HCl		1984	20, 321
oxiconazole nitrate		1983	19, 322
terconazole		1983	19, 324
tioconazole		1983	19, 324
butoconazole	ANTIFUNGAL, TOPICAL	1986	22, 318
cloconazole HCl		1986	22, 318
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
cetirizine HCl		1987	23, 331
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
benazepril hydrochloride		1990	26, 299
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
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
guanadrel sulfate		1983	19, 319

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isradipine		1989	25, 315
ketanserin		1985	21, 328
lisinopril		1987	23, 337
manidipine hydrochloride		1990	26, 304
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
trimazosin HCl		1985	21, 333
AF-2259	ANTIINFLAMMATORY	1987	23, 325
amfenac sodium		1986	22, 315
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
felbinac		1986	22, 320
halometasone		1983	19, 320
hydrocortisone aceponate		1988	24, 304
hydrocortisone butyrate propionate		1983	19, 320
mometasone furoate		1987	23, 338
piketoprofen		1984	20, 322
pimaprofen		1984	20, 322
prednicarbate		1986	22, 325
pravastatin	ANTILIPIDEMIC	1989	25, 316

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artemisinin	ANTIMALARIAL	1987	23, 327
halofantrine		1988	24, 304
mefloquine HCl		1985	21, 329
alpiropride	ANTIMIGRAINE	1988	24, 296
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
flutamide		1983	19, 318
fotemustine		1989	25, 313
idarubicin hydrochloride		1990	26, 303
interleukin-2		1989	25, 314
lonidamine		1987	23, 337
mitoxantrone HCl		1984	20, 321
nilutamide		1987	23, 338
pirarubicin		1988	24, 309
ranimustine		1987	23, 341
toremifene		1989	25, 319
vinorelbine		1989	25, 320
ivermectin	ANTIPARASITIC	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
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

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cilostazol		1988	24, 299
enoxaparin		1987	23, 333
ethyl icosapentate		1990	26, 303
ozagrel sodium		1988	24, 308
indobufen		1984	20, 319
picotamide		1987	23, 340
limaprost		1988	24, 306
flutropium bromide	ANTITUSSIVE	1988	24, 303
levodropropizine		1988	24, 305
benexate HCl	ANTIULCER	1987	23, 328
enprostil		1985	21, 327
famotidine		1985	21, 327
irsogladine		1989	25, 315
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
foscarnet sodium	ANTIVIRAL	1989	25, 313
ganciclovir		1988	24, 303
rimantadine HCl		1987	23, 342
zidovudine		1987	23, 345
buspirone HCl	ANXIOLYTIC	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
ipriflavone		1989	25, 314

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bucladesine sodium	CARDIOSTIMULANT	1984	20, 316
denopamine		1988	24, 300
dopexamine		1989	25, 312
enoximone		1988	24, 301
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
trientine HCl	CHELATOR	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
cinitapride	GASTROPROKINETIC	1990	26, 301
cisapride		1988	24, 299
erythropoietin	HEMATOPOETIC	1988	24, 301
malotilate	HEPATROPROTECTIVE	1985	21, 329
buserelin acetate	HORMONE	1984	20, 316
goserelin		1987	23, 336
leuprolide acetate		1984	20, 319
nafarelin acetate		1990	26, 306
somatropin		1987	23, 343
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
acetohydroxamic acid	HYPOAMMONURIC	1983	19, 313

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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
thymopentin		1985	21, 333
lentinan	IMMUNOSTIMULANT	1986	22, 322
pegademase bovine		1990	26, 307
schizophyllan		1985	22, 326
ubenimex		1987	23, 345
cyclosporine	IMMUNOSUPPRESSANT	1983	19, 317
mizoribine		1984	20, 321
muromonab-CD3		1986	22, 323
sulbactam sodium	β-LACTAMASE INHIBITOR	1986	22, 326
afloqualone	MUSCLE RELAXANT	1983	19, 313
eperisone HCl		1983	19, 318
tizanidine		1984	20, 324
naltrexone HCl	NARCOTIC ANTAGONIST	1984	20, 322
tinazoline	NASAL DECONGESTANT	1988	24, 312
timiperone	NEUROLEPTIC	1984	20, 323
bifemelane HCl	NOOTROPIC	1987	23, 329
choline alfoscerate		1990	26, 300
exifone		1988	24, 302
idebenone		1986	22, 321
indeloxazine HCl		1988	24, 304
levacecarnine HCl		1986	22, 322
nizofenzone fumarate		1988	24, 307
oxiracetam		1987	23, 339
epoprostenol sodium	PLATELET AGGREG. INHIBITOR	1983	19, 318
gestodene	PROGESTOGEN	1987	23, 335
nomegestrol acetate		1986	22, 324
norgestimate		1986	22, 324
promegestrone		1983	19, 323

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alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24, 297
nafamostat mesylate		1986	22, 323
adrafinil	PSYCHOSTIMULANT	1986	22, 315
surfactant TA	RESPIRATORY SURFACTANT	1987	23, 344
APSAC	THROMBOLYTIC	1987	23, 326
alteplase		1987	23, 326
tiopronin	UROLITHIASIS	1989	25, 318
clobenoside	VASOPROTECTIVE	1988	24, 300
cadexomer iodine	WOUND HEALING AGENT	1983	19, 316
epidermal growth factor		1987	23, 333