ANNUAL REPORTS IN MEDICINAL CHEMISTRY Volume 21

Sponsored by the Division of Medicinal Chemistry of the American Chemical Society

Editor-in-Chief: ***DENIS M. BAILEY***

STERLING–WINTHROP RESEARCH INSTITUTE
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PREFACE

Volume 21 of *Annual Reports in Medicinal Chemistry* contains 31 chapters in seven sections: CNS Agents, Pharmacodynamic Agents, Chemotherapeutic Agents, Metabolic Diseases and Endocrine Function, Topics in Biology, Topics in Chemistry and Drug Design, and Worldwide Market Introductions. The last of these sections, first introduced in Volume 19, continues to be a popular adjunct to the more classic topics of this serial.

In this volume, the Section Editors have been granted latitude in addressing the process and future of drug discovery, as well as the more traditional reporting of the last year's events. Thus, in each section there appear chapters that attempt to add the perspective of future opportunity to the established accomplishments of the past and present. These chapters include potential mechanistic approaches to the discovery of safer neuroleptics (chapter 1), and agents for the treatment of acute ischemic stroke (chapter 11), atherosclerosis (chapter 19), and autoimmune diseases (chapter 20). Issues confronting discovery research in the maturing antibiotic area are addressed in chapter 12. In Section VI, the utility of powerful tools to probe molecular interactions (chapter 28) and structure (chapter 30) is described.

Because of their scientific importance and rapidly evolving therapeutic potential, various aspects of molecular biology have been covered in several chapters. In some instances these general chapters extend across therapeutic areas. For example, chapters 16 and 22 discuss the oncogenic potential of peptide growth factors, the former from a more medical viewpoint and the latter from a biochemical perspective.

Updated in this volume are the areas of anxiolytics, anticonvulsants, sedative-hypnotics, analgesics, antihypertensives, pulmonary, antiarrhythmic and antiallergy agents, and anti-infectives. In addition, the potential medical utilities of atrial natriuretic factor and colony stimulating factors are reviewed. The current status of bioisosterism in drug design and the use of computers in planning organic syntheses are discussed in Section VI.

In its continuing effort to vitalize the format of *Annual Reports*, the editorial board has elected to experiment with the addition of an in-depth review of an area globally important to drug discovery. In this year's volume the concept and function of receptors are extensively covered in the section on Topics in Biology (chapter 21).

It has been a pleasure to work with the Section Editors and contributors to this volume. They have, indeed, generated and assembled a fine collection of reports. As usual, this volume could not have been completed without the assistance of Martha Johnson, to whom I am most grateful.

Denis M. Bailey
Rensselaer, New York
May 1986

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Section I — CNS Agents

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Chapter 1. Atypical Antipsychotic Agents

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Introduction and Background — While neuroleptic drugs continue to represent the best form of treatment for schizophrenia, they still possess two major weaknesses. First, as many as one-third of the patients suffering from this disorder, particularly those with a preponderance of negative symptoms, do not respond to neuroleptic treatment.[1,2] Second, all neuroleptics currently approved in the United States produce extrapyramidal side effects (EPS).[2] The most common forms of EPS are Parkinsonism, dystonia, akathisia and tardive dyskinesia (TD). The first three types occur early in the course of neuroleptic treatment and can usually be ameliorated. The fourth form, TD, develops only after months or years of therapy. It can be life-threatening and is very difficult to reverse even after termination of neuroleptic treatment.[3] Both acute and chronic EPS are believed to result from blockade of dopamine (DA) receptors in the neostriatum of the brain.[2,3] The acute symptoms are likely due to a decrease in striatal DA functioning. On the other hand, TD appears to result from the ability of the striatal DA system to overcome the neuroleptic blockade and ultimately reestablish more normal levels of neurotransmission. Since the striatal DA receptors have been made supersensitive by chronic blockade, the restored dopaminergic stimulation produces the abnormal movements of TD.[3]

In recent years efforts have been focused on the need for drugs with greater clinical efficacy, and/or reduced liability to produce EPS, especially TD. Drugs which possess this clinical profile, or are predicted to exhibit it based on preclinical data or mechanistic hypotheses, have been termed "atypical antipsychotics."

Putative Mechanisms Subserving Atypical Activity — The pharmacological mechanisms most often suggested as contributing to atypical antipsychotic activity (sometimes in combination) are described in this section.

Selective antagonism of limbic DA receptors — The consensus is that antipsychotic drugs ameliorate psychosis by blocking the DA receptors in limbic regions of the brain (e.g., nucleus accumbens, olfactory tubercle, frontal cortex) while EPS result from the concomitant blockade of DA receptors in the striatum.[4,5] It follows that a drug which preferentially blocks limbic DA receptors might have a lower propensity to produce EPS.[6] Behavioral tests are generally used to identify DA antagonists which are selective for the limbic system. Inhibition of the enhanced locomotor behavior resulting from the administration of dopamine agonists is thought to result from limbic (i.e., nucleus accumbens) DA receptor blockade, while reversal of the stereotyped behavior produced by higher doses of DA agonists appears more related to the inhibition of striatal DA receptors.[7-9] Likewise, the blockade of striatal DA receptors seems important in mediating catalepsy.[10-12] Therefore, limbic DA receptor blockade, and presumably antipsychotic activity, would be indicated by the inhibition of locomotor activity, while decreased striatal DA receptor blockade, and reduced EPS liability, would be predicted by little or no inhibition of stereotypy, and the absence of catalepsy. The absence of supersensitivity to DA agonists in behavioral paradigms subserved by striatal mechanisms, following chronic administration of a putative antipsychotic, may be indicative of reduced TD liability; the

neurochemical correlate of such behavioral measures is the absence of striatal DA receptor proliferation.[3]

Antimuscarinic Activity — Blockade of muscarinic receptors in the striatum produces behavioral effects opposite to those caused by blockade of DA receptors. Thus, antimuscarinic drugs, such as scopolamine, potentiate the stereotypy induced by amphetamine, while atropine reverses haloperidol-induced catalepsy.[8,13] The antimuscarinic potencies of antipsychotic drugs are inversely correlated with their liabilities to produce EPS in man.[14,15] This supports the hypothesis that concomitant antimuscarinic activity will attenuate the effects of DA antagonists in the striatum.

Gamma-Aminobutyric acid (GABA) agonist activity — Chronic administration of neuroleptics causes many changes in brain GABAergic systems, including a decrease of GABA turnover in limbic and striatal regions, decreased activity of glutamic acid decarboxylase (GAD) in the substantia nigra and a proliferation of nigral GABA receptors. [16-18] These changes in the GABAergic system could contribute to TD in two ways. First, since the GABAergic striatonigral pathway affects neurotransmission from the striatum to other brain regions, a proliferation of nigral GABA receptors could facilitate the passage of information down this pathway, thereby enhancing the effects of striatal DA receptor activation and (ultimately) inducing TD.[19] Conversely, the decrease in nigral GAD levels and GABA turnover could itself lead to TD.[20] It has been suggested that the combination of a GABA agonist with a dopamine antagonist could prevent these processes, either by preventing nigral GABA receptor proliferation or by maintaining tonic GABAergic activation, and that such a combination would have a reduced TD liability.[19,20] However, since GABA agonists do not improve psychosis (and may even worsen it), drugs with this profile would not be expected to have increased clinical efficacy.[21,22]

Noradrenergic antagonist activity — Norepinephrine (NE)-containing brain systems have also been implicated in the etiology of schizophrenia. Schizophrenic patients, particularly those with the chronic form of the disease, have elevated levels of NE and its major metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), in some brain areas as well as elevated NE levels in their cerebrospinal fluid.[23-25] These results point to altered noradrenergic system functioning in schizophrenia, interpreted as increased activity by some,[23,26] and decreased activity by others.[24,25] Altered noradrenergic function might contribute directly to psychosis or the effects could be indirect *via* modulation of the DA system. The latter possibility is supported by experimental data showing an interaction of noradrenergic and dopaminergic systems in a number of behaviors including amphetamine-induced hyperactivity, amphetamine-induced stereotypy, conditioned avoidance, aggression, feeding, motor activity and catalepsy.[27-30] Support for a beneficial effect of NE system antagonism in schizophrenia has also come from clinical trials of propranolol in this disorder. Most studies have shown benefit with propranolol alone or in combination with a neuroleptic,[31-37] although some have failed to demonstrate efficacy.[38-40]

Serotonin (5-HT) antagonist activity — 5-HT antagonists can reverse some of the biochemical and behavioral effects of neuroleptics. Thus, neuroleptic-induced catalepsy is prevented or reduced by 5-HT receptor antagonists (cyproheptadine, mianserin, or methysergide), or by lesions of the serotonergic system.[41-44] In addition, methysergide, mianserin and cinanserin block the increase in DA turnover produced by haloperidol in the striatum.[45] Cyproheptadine and methysergide also reverse fluphenazine's inhibitory effect on the locomotor behavior produced by direct administration of DA in the nucleus accumbens: on this basis, it is unlikely that 5-HT antagonists would improve antipsychotic efficacy,[46] although serotonergic overactivity has been associated with some syndromes of schizophrenia.[47]

Other mechanisms — A drug which does not directly block DA receptors, but produces some of the electrophysiological, biochemical or behavioral effects common to clinically active neuroleptics, might possess antipsychotic activity without the risk of EPS. The behavioral tests usually employed to search for this activity, such as conditioned avoidance behavior, intracranial self-stimulation and unconditioned behaviors, are independent of *a priori* DA receptor blockade.[48] However, some investigators have also employed DA-induced behaviors including the climb-

ing and aggressive behaviors induced by apomorphine.[48] Several neuropeptides, including cholecystokinin (CCK), neurotensin and des-tyrosine-gamma-endorphin (DTGE) produce effects on DA turnover or behavior that simulate those of neuroleptics without binding directly to DA receptor sites.[49-55] However, clinical trials of DTGE have shown only modest, short-lived antipsychotic activity, while trials of CCK have failed to demonstrate activity reproducibly.[56-58] Recent studies have described a putative endogenous peptide which binds to the sigma opiate receptor.[59] Since agonists which bind to this receptor typically have potent psychotogenic activity (e.g., phencyclidine, PCP),[60] it is thought that an antagonist might possess antipsychotic efficacy.[61] Again, blockade of DA receptors would be circumvented.

Clozapine: The Prototypical Atypical — The clinical effectiveness of clozapine has been documented in a large number of studies which, almost without exception, have noted the virtual absence of EPS.[62-67] Furthermore, clozapine has often been rated as more efficacious than classical agents such as chlorpromazine.[68-75] The extensive efforts to elucidate the mechanisms underlying clozapine's unique and desirable clinical profile can be analyzed with regard to the six putative rationales for atypical activity set forth in the previous section.

Selective antagonism of limbic DA receptors — Selective activity in limbic systems has been noted in the behavioral, biochemical, neurochemical and electrophysiological effects of clozapine. In behavioral tests, clozapine inhibits the locomotor stimulation but not the stereotypy induced by apomorphine, and does not produce catalepsy.[76,77] Biochemically, clozapine produces a greater enhancement of DA turnover (at least with optimal treatment parameters) in limbic than striatal brain regions, while conventional neuroleptics produce an equal or greater effect in the striatum.[78-82] Likewise, while classical drugs (with the exception of thioridazine) acutely produce an increase, and chronically a decrease, in the firing rates of DA-containing neurons projecting to both limbic and striatal brain regions, clozapine produces these effects preferentially or exclusively in neurons projecting to the limbic region.[83,84] Furthermore, clozapine selectively binds to limbic compared to striatal brain regions, whereas conventional neuroleptics do not reproducibly show any preference or are striatally selective.[85-87] However, selective binding may not occur in man.[88]

While the consensus is that clozapine does induce supersensitivity on chronic administration, this effect is believed to apply more to behaviors mediated by limbic, rather than striatal systems.[89-92] Also, in contrast to conventional neuroleptics, chronically administered clozapine does not cause a proliferation of striatal DA receptor binding sites.[92,93] These data suggest that clozapine produces a weak striatal DA receptor blockade, and are consistent with the reported absence of TD in patients treated for extended periods of time with this drug.

Antimuscarinic activity — Clozapine is an anticholinergic agent with high affinity for muscarinic cholinergic receptors.[77] A study of the effect of clozapine (intra-accumbens administration) on amphetamine-induced stereotypy concluded that clozapine's potent anticholinergic activity modulates its mesolimbic dopaminergic properties: the combination of clozapine with a cholinergic agonist effectively antagonized stereotypy, whereas clozapine in combination with an anticholinergic agent significantly potentiated the stereotyped behaviors.[94] Muscarinic binding in the mouse brain (cortex, hippocampus, and striatum) is increased upon chronic administration of clozapine, consistent with its anticholinergic activity; this up-regulation of muscarinic sites may offer protection against TD.[95] The anticholinergic effects of clozapine might also be responsible for its preferential elevation of DA turnover in limbic areas, since this effect can be mimicked by co-administration of haloperidol and an anticholinergic drug.[78]

GABA agonist activity — Unlike haloperidol or chlorpromazine, clozapine does not up-regulate GABA receptors in the substantia nigra following chronic administration. It has been speculated that the anticholinergic activity of clozapine interferes with the activation of nigrostriatal pathways required for the suppression of GABA transmission, thus preventing the development of supersensitive GABA receptors "downstream" from dopamine receptors.[17] However, clozapine may exert a more direct effect on GABA receptors since it has been found to facilitate the binding of ^{3}H-flunitrazepam to mouse brain *in vivo* (an effect produced by GABA agonists).[96]

Noradrenergic antagonist activity — Chronic administration of haloperidol to rats was shown to inactivate both A9 (striatal) and A10 (limbic) neurons in an electrophysiological study, whereas clozapine under the same treatment protocol inactivated A10 cells selectively. Chronic co-administration of haloperidol and an alpha$_1$ antagonist drug (prazosin) mimicked the chronic clozapine effect. The combination of haloperidol with an alpha$_2$ antagonist did not produce clozapine-like A10 selectivity. Thus, alpha$_1$ antagonism may be responsible in part for some of clozapine's selective limbic effects.[97]

5-HT antagonist activity — The 5-HT$_2$ antagonist properties of clozapine have been demonstrated by several investigators. In addition to inhibiting 5-HT$_2$ receptor binding, clozapine blocks the ability of rats to discriminate quipazine, a direct 5-HT receptor agonist.[98] In another set of experiments clozapine blocked the potentiation of apomorphine-induced hypermotility by LSD in the rat,[99,100] an effect whose locus of action appears to be the median raphe nucleus. Chronic administration of clozapine causes adaptive changes in the serotonin system: 5-HT$_2$ receptors (frontal cortex) are down-regulated by 63% in the rat.[101]

Other mechanisms — ^{3}H-Clozapine has been utilized in several binding experiments in efforts to determine whether this drug might label a novel, pharmacologically relevant site. Much of the binding was to muscarinic receptors, and the remaining specific binding was largely non-dopaminergic.[102-104] To date there is no conclusive evidence for a unique, functionally significant "clozapine binding site."

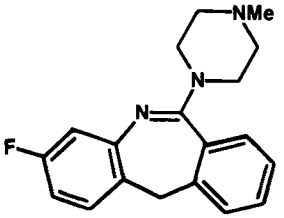
Clinically Tested Putative Atypical Agents — In addition to clozapine, a number of drugs from diverse structural classes have been reported to exhibit atypical clinical activity. The substituted benzamides (e.g., sulpiride) have not been included in this review for two reasons. First of all, controversy still exists as to whether sulpiride, the most well-studied agent from this structural class, truly displays atypical activity: it has been difficult to determine if, in fact, sulpiride, compared to classical neuroleptics, possesses roughly equivalent EPS liability when administered in doses high enough to be antipsychotic.[105] Sulpiride is a relatively weak antagonist at the D$_2$ DA receptor but has no effect on NE, 5-HT, ACh, histamine, or GABA receptors.[106-108] Chronic administration of high doses of sulpiride does produce behavioral and biochemical supersensitivity to DA agonists.[109] Very high doses of this agent given acutely have been shown to induce catalepsy.[87]

Secondly, the newer benzamides are, like sulpiride, selective D$_2$ blockers. Drugs such as etoclopride and raclopride differ from sulpiride primarily in terms of greater potency as DA antagonists.[110,111] Since little or no clinical information regarding the potent benzamides is available at the present time, it will be best to review these drugs after a number of independent studies of efficacy and tolerance have been published.

Fluperlapine (1) — This drug was effective in several open and single blind trials and did not produce EPS.[112-117] Like clozapine, its atypical activity could be due to any of a number of mechanisms.[118] Fluperlapine may be a selective antagonist at limbic DA receptors since it is weak as a blocker of apomorphine stereotypy and does not induce catalepsy (both striatal effects). However, its activity against DA agonist-induced hyperlocomotion has apparently not been examined, so that no limbic versus striatal comparison of behavioral effects can be made. In receptor-binding studies fluperlapine shows weak affinity for the DA receptor but binds potently to muscarinic, 5-HT$_2$ and alpha$_1$-adrenergic sites. While fluperlapine is anticholinergic *in vivo*, and increases striatal levels of the DA metabolite, homovanillic acid (HVA), it is much less effective at increasing levels of the norepinephrine metabolite, MHPG, in the brain system. Thus, some combination of antidopaminergic, antimuscarinic or antiserotonergic activities may be responsible for the atypical psychotherapeutic profile of this drug.

Rimcazole (2) — This agent has been tested in several open clinical trials where it has reportedly shown efficacy with minimal EPS.[119-123] The antipsychotic activity of rimcazole appears not to involve DA receptors directly: it is extremely weak as an inhibitor of D$_2$ receptor binding.[124] Rimcazole may act as a selective DA antagonist in the limbic system since it increases the concentrations of the DA metabolites, HVA and 3,4-dihydroxyphenylacetic acid (DOPAC), in the prefrontal cortex but not in the striatum.[125] In addition, it does not block apomor-

phine or amphetamine-induced stereotypy, nor does it induce catalepsy.[126] Rimcazole does block apomorphine-induced climbing in mice and inhibits several forms of aggressive behavior, but does not inhibit conditioned avoidance behavior.[126] The only potent binding interaction of rimcazole is at the site labeled by ^{3}H-SKF-10047.[127] While this observation may suggest sigma receptor antagonism as a mechanism of action, rimcazole did not inhibit binding of the putative sigma receptor agonist and psychotogen, ^{3}H-PCP.[127]



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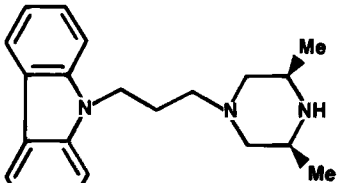

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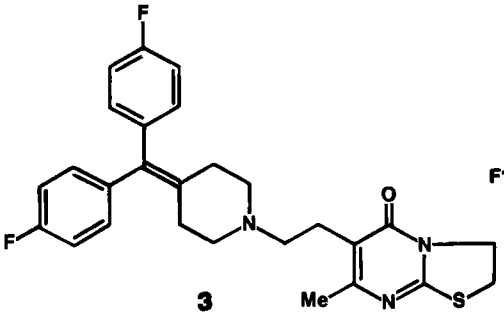
Ritanserin (3) — This drug has shown clinical efficacy with reduced EPS liability in both open and controlled clinical trials (with or without an added neuroleptic).[128-130] Ritanserin is a very potent 5-HT_2 antagonist. By comparison, it is considerably weaker as a D_2 receptor blocker. Despite its binding potency at DA receptor sites, ritanserin does not alter levels of DA or its metabolites, except at very high doses. It is also ineffective in antagonizing behaviors produced by dopaminergic stimulation. Ritanserin has moderately high potencies at alpha$_1$- and alpha$_2$-adrenergic binding sites, which may contribute to its atypical profile.[131]



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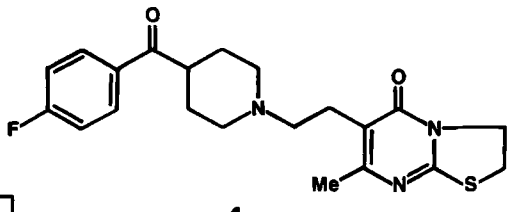

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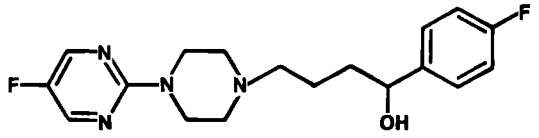
Setoperone (4) — This drug has been tested in open clinical trials in patients with mainly negative schizophrenic symptoms.[132] It was efficacious in these studies with a reduced liability to produce EPS. Setoperone inhibits DA and 5-HT_2 binding with approximately equal potencies, and blocks the *in vivo* effects of tryptamine, as well as those of amphetamine and apomorphine (including apomorphine-induced stereotypy).[133-134] It is devoid of anticholinergic activity.[134]

Other Putative Atypical Antipsychotics — This section will discuss those agents which have been purported to possess atypical antipsychotic activity based on preclinical biochemical, neurochemical or behavioral data. We have omitted from this review the selective D_1 DA antagonist, Sch 23390, which has been recently described elsewhere.[135] A functional interrelationship between D_1 and D_2 receptors has been noted by several investigators.[136,137] Like conventional neuroleptics, Sch 23390 has been reported to induce catalepsy,[138] although there has been some disagreement as to whether the observed immobile state is a true haloperidol-like catalepsy.[135] Also, this drug has been found to potentiate haloperidol-induced dyskinesias in sensitized squirrel monkeys.[139] Although Sch 23390 has been shown to differ from classical agents by virtue of a lack of direct effects on D_2 receptors,[140,141] it is difficult to categorize this drug as atypical in the absence of clinical data.

BMY-14802 (5) — The putative atypical activity of this compound is based on its neuroleptic-like effects in electrophysiological, biochemical and behavioral tests in the absence of *in vitro* DA receptor blockade. BMY-14802 lacks affinity for any binding sites thus far examined, including dopaminergic (D_2), muscarinic, serotonergic or adrenergic (alpha$_1$- or beta-).[142-144] However, it inhibits conditioned avoidance behavior, apomorphine-induced stereotypy and amfonelic acid-

induced locomotion, and does not produce catalepsy.[142-144] Also, like neuroleptics, it increases DA cell firing and DA turnover in the striatum when administered systemically.[143]



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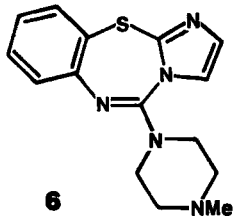

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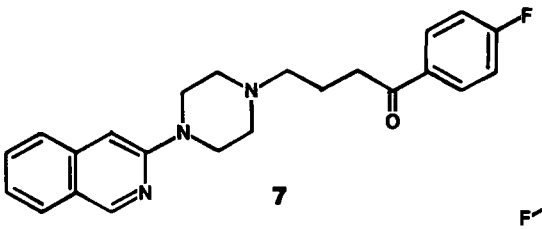
CGS-10746B (6) — This compound displays neuroleptic-activity without *in vitro* DA receptor blockade.[145] It blocks neither D_2 nor muscarinic binding sites. However, CGS-10746B inhibits conditioned avoidance behavior and intracranial self-stimulation, and does not produce the acute dyskinetic syndrome in monkeys. Like other neuroleptics, it produces an increase in striatal levels of the DA metabolite, DOPAC; however, this increase is small. CGS-10746B also possesses weak alpha-adrenergic antagonist properties.

Cinuperone (7) — This agent selectively inhibits DA-dependent behaviors mediated by the limbic system.[146,147] Thus, cinuperone inhibits methamphetamine-induced locomotor behavior with higher potency than it blocks amphetamine-induced stereotypy, and does not induce catalepsy. In addition, this drug inhibits apomorphine-induced climbing, conditioned avoidance behavior, intra-cranial self stimulation and apomorphine-induced turning in lesioned animals. Cinuperone is a potent inhibitor of DA (D_2) and NE ($alpha_1$), but not muscarinic binding.



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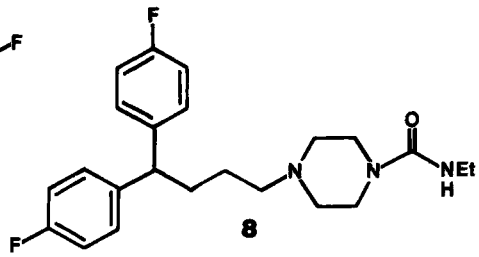

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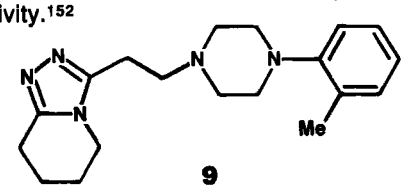
Amperozide (8) — This compound is selective for presynaptic DA terminals in the limbic system and is also a potent 5-HT antagonist.[148] In principle, attenuation of DA systems *via* presynaptic mechanisms could result in reduced EPS liability. However, although behavioral data suggest that amperozide acts selectively at presynaptic DA terminals, it apparently does not affect DA synthesis.[149] Amperozide inhibits the locomotor behavior, but not the stereotypy caused by amphetamine, and does not cause catalepsy. These findings provide behavioral evidence for limbic selectivity.[149,150] The potent 5-HT_2 receptor binding and decreased 5-HT cell firing produced by amperozide suggest functional antagonism of the serotonergic system.[150] However, these antagonist properties may be partially mollified by the ability of this agent to inhibit 5-HT uptake.[150] Amperozide also has weak effects on noradrenergic binding ($alpha_1$- and $alpha_2$-), uptake and cell firing.[150]

Dapiprazole (9) — Dapiprazole is reported to be neuroleptic-like but devoid of direct DA receptor-blocking activity. However, the claim for lack of DA receptor inhibition rests on the failure of this compound to inhibit apomorphine stereotypy or to induce catalepsy.[151] These activities are also absent in some known DA receptor antagonists (e.g., clozapine). Dapiprazole does block amphetamine locomotor behavior, which indicates selectivity for the limbic system, and also inhibits conditioned avoidance behavior.[151] It is a potent noradrenergic blocker (alpha-, but not beta-) and a somewhat less potent inhibitor of serotonin, but is devoid of anticholinergic activity.[152]



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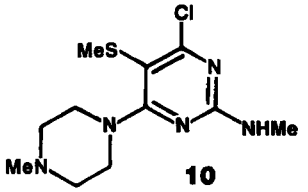

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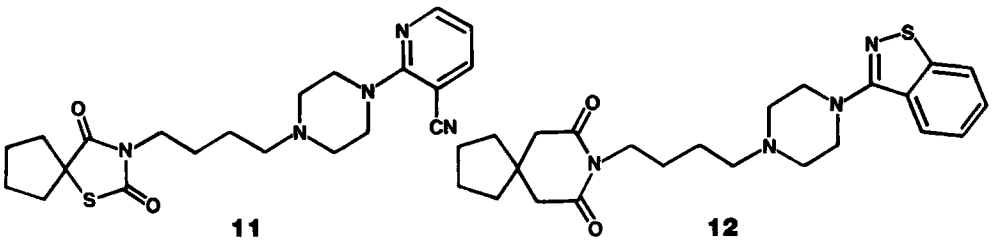
Mezilamine (10) — This agent is classified as an atypical antipsychotic on the basis of its selectivity for the limbic DA system and its weak induction of catalepsy.[153,154] Mezilamine inhibits DA (D_2) binding *in vitro* (^{3}H-haloperidol) more potently in the olfactory tubercle than in the striatum. It also increases DA turnover to a greater extent in the nucleus accumbens than in the striatum.[153,154] However, some of these effects may be attributable to alpha-adrenergic mechanisms.[153,154] Mezilamine does not inhibit muscarinic cholinergic binding and does not display GABA agonist activity *in vivo*.[155] Like other neuroleptics, mezilamine blocks apomorphine-induced stereotypy and yawning, and conditioned avoidance behavior.[155,156]



11 12

MJ-13980-1 (11) — On the basis of its failure to produce catalepsy on acute administration, or striatal supersensitivity on chronic administration, it is predicted that this compound will show decreased EPS liability.[157,158] MJ-13980 does, however, block apomorphine-induced stereotypy and striatal ^{3}H-spiroperidol binding, which indicates inhibition of the striatal DA system.[158] It is devoid of antimuscarinic activity.[157]

Tiasperone (12) — Tiasperone is weakly cataleptogenic and fails to induce striatal DA receptor binding site proliferation on chronic administration; it does, however, inhibit conditioned avoidance behavior and amfonelic acid-induced locomotor behavior.[159,160] Tiasperone is more effective in preventing firing of DA-containing neurons which project to limbic than to striatal areas, following chronic administration.[161] The above effects are consistent with limbic selectivity. By contrast tiasperone is as potent an inhibitor of apomorphine-induced stereotypy (a striatally-mediated behavior) as of amfonelic acid-induced hyperlocomotion.[159,160] Receptor-binding studies show that **12** has high affinity for DA (D_2), adrenergic ($alpha_1$) and 5-HT receptors: it does not bind to muscarinic receptors.[160]

Summary — We have described many of the compounds under development which are claimed to be atypical antipsychotic drugs free from the major deficiencies of currently marketed agents. However, it will take many years of clinical studies before it can be determined, which, if any, of these are truly "atypical" antipsychotics, both from the standpoint of improved efficacy and reduced EPS liability.

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Chapter 2. Anxiolytics, Anticonvulsants and Sedative-Hypnotics

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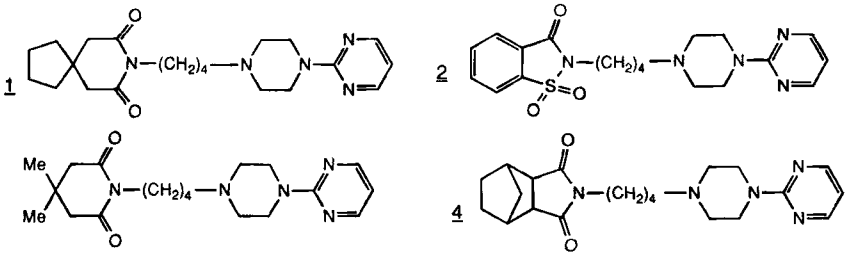
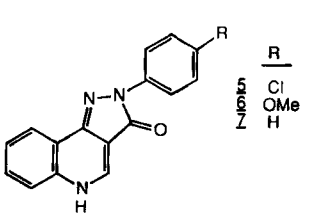
Introduction - Public interest in anxiety has continued to increase, with over 13 million individuals in the U.S.A. being affected by this disorder.[1] At the preclinical level, the emphasis has been on attempts to separate the various pharmacological actions of the benzodiazepines (i.e. anxiolytic, sedative, anticonvulsant and muscle relaxant), into distinct processes mediated via different receptor subtypes, although this approach is more molecular than physiological.[2-5] Concurrently, based on the activity of buspirone (1)[6] and related compounds (2)-(4),[7-9] the mechanistic emphasis has switched to the role of serotonin in the

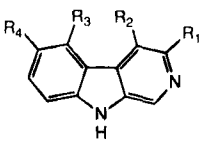


etiology of anxiety.[9] Compound 1 does not interact with central benzodiazepine (BZ) receptors, is devoid of sedative and muscle relaxant actions and does not potentiate alcohol.[6] There have been new developments pertaining to the putative endogenous ligand for the central receptor[10] and the functional role of peripheral BZ receptors (PBZR).[11,12]

Benzodiazepine receptor ligands - Compounds directly interacting with the central BZ receptor include the pyrazoloquinoline, CGS 9896 (5), a non-sedating anxiolytic devoid of muscle relaxant activity and with reduced propensity to potentiate alcohol,[13] which continues to be distinguished from typical BZ anxiolytics in various animal models.[14-17] A close analog, CGS 9895 (6) is a potent BZ partial agonist[18] and a selective antagonist of some anticonvulsants, as is β-CMC (8).[19] Classical BZ

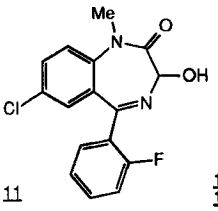


	R
5	Cl
6	OMe
7	H



	R_1	R_2	R_3	R_4
8	NHCOOMe	H	H	H
9	COOEt	CH_2OMe	H	OCH_2Ph
10	COOEt	CH_2OMe	OCH_2Ph	H

structures also remain of interest. SAS 646 (11) and the 'hetrazepine', brotizolam (WE 941, 12) have been introduced as anxiolytics in Europe. Metaclazepam, (KC-2547, 14) is a putative anxiolytic with less muscle relaxant properties than diazepam or bromazepam.[20]



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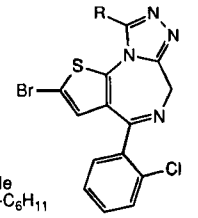
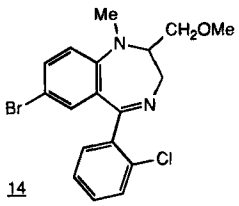


12 R=Me
13 R=c-C_6H_{11}



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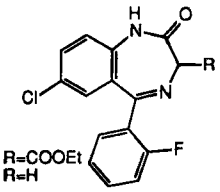
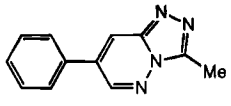


15 R=COOEt
16 R=H



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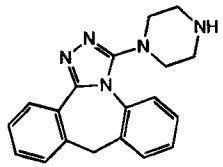

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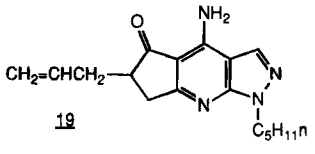
Loflazepate (CM 6912, 15) is a potent inhibitor of radioligand binding to the central BZ receptor, although a metabolite, (CM 7116, 16), is more effective in potentiating the suppression of guinea pig cerebellar Purkinje cell firing by GABA.[21] A series of 1-azacycloalkyl BZs has been reported to have antianxiety/antidepressant actions.[22] The rauwolfia alkaloid, raubasine, can inhibit BZ binding to the receptor competitively and has anticonvulsant activity in vivo.[23] SR 95195 (17) has been reported to be a BZ antagonist.[24] Pitrazepin (18), a potent competitive GABA-A receptor antagonist, is non-selective and binds to glycine receptors at equivalent concentrations.[25] The quinoline partial agonist PK 8165, has anxiolytic activity in humans.[26] Compound 19 is active in binding assays and animal models.[27] "Hetrazepine" 20 has anticonflict, but no anticonvulsant, activity.[28] Ro 15-3505 (21)[29] and compound 22[30] are BZ antagonists.



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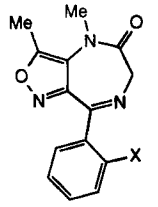
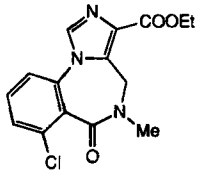


20 X=Cl, CF_3



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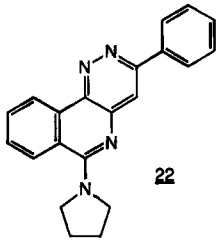

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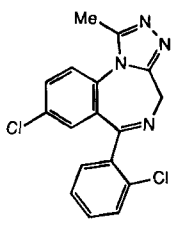
BZ Receptor Subtypes - Controversy still surrounds the delineation of BZ receptor subtypes, as assessed by ligand binding studies, and their pharmacological relevance.[3,4] The temperature dependence of BZ binding has questioned the physiological relevance of the minutiae generated in bind-

ing studies,[31,32] while the anomalous binding properties of triazolam (23) have been linked to its hypnotic actions.[31] New studies have cast doubt on the earlier suggestion that the BZ-1 subset of binding sites constitutes the anxiolytic receptor.[4] The BZ-1 sites are enriched in the cerebellum, a brain region more involved in motor than in cognitive function. By default, the hippocampal BZ-2 receptor was deemed responsible for the ancillary pharmacology of the BZs. The hypnotic, zolpidem (SL 80.0750, 24) binds selectively to BZ-1 receptors.[33-34] BZ-1 and BZ-2 receptors are each located at pre- and postsynaptic sites.[35,36] The chloride channels associated with the central BZ receptor complex appear to exist in multiple forms, their number being eight- and four-fold higher than the respective numbers of BZ and GABA-A receptors.[37] Other studies have shown that the functional state of the chloride channel is markedly dependent on GABA.[38-40]

Monoclonal antibodies to the bovine central BZ receptor complex interact with four antigenic determinants on the receptor.[41] The antibodies cross-reacted with rat and human brain receptor complexes but did not affect BZ binding to these receptors. The molecular weights of the BZ binding site in the central receptor complex, and the picrotoxinin/barbiturate binding site, are 51K and 138K daltons, respectively, as determined by radiation inactivation studies.[42] When isolated by CHAPS solubilization, the BZ receptor from rat brain has a Stokes radius of 7.7 nm and is GABA-sensitive in terms of its binding characteristics; the complex isolated using Triton X-100 has a radius of 6.0 nm and is insensitive to GABA.[43]

The use of protein modifying reagents has further confirmed that the binding site for suriclone on the BZ receptor complex is distinct from that for the BZs,[44] while avermectin interactions with the complex involve a binding site distinct from that for barbiturates.[45] BZ receptors are present in the pituitary gland.[46]



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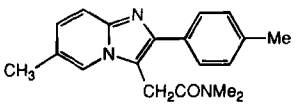

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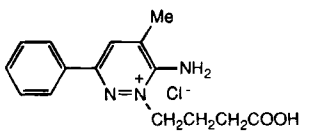

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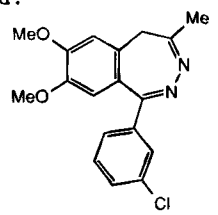

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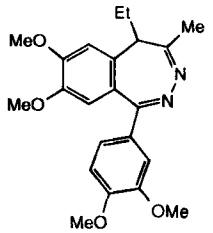

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Preliminary reports have shown that the BZ receptor can undergo phosphorylation and glycosidases can alter BZ ligand binding to the receptor.[47] A comparison of the X-ray structure of the BZ antagonist Ro 15-1788, the pyrazoloquinoline inverse agonist CGS 8216 (7) and members of the β-carboline series has allowed delineation of the receptor site topography for both agonists and antagonists: the model suggested on the basis of this limited SAR has seven conformationally mobile binding points.[48] [^{11}C]-Ro 15-1788 has been used to map BZ receptor sites in human brain using positron emission tomography.[49,50] The GABA effector, valproate has minor tranquilizer activity,[51] while GYKI-51 189 (25), a BZ that does not bind to the central BZ receptor, can enhance binding of GABA to a low affinity site.[52] This compound is also more active as an anxiolytic/antidepressant than its analog, tofizopam 26. The arylaminopyridazine derivative of GABA, SR 95103 (27), is a potent inhibitor of

GABA-stimulated BZ binding[53] thereby reducing seizure threshold.[54] The glucocorticoids, corticosterone and pregnenolone sulfate, enhance binding to the GABA-A receptor.[55] Other compounds affecting ligand binding to the BZ receptor complex are cannabinoids,[56] methylimidazoleacetic acid,[57] the antagonist compound 7,[58] as well as several beta-carboline related entities.[59-61] The involvement of GABA in the anxiolytic, as opposed to the sedative, actions of BZs has been questioned as has the predictive value of the 'GABA-shift'.[62,63] Studies of the effects of GABA on the binding of the "hetrazepines" (vide supra) to central BZ receptors have suggested that facilitation of GABAergic transmission may be related to the hypnotic, rather than the anticonflict or anticonvulsant actions of the BZs.[64] In this context, binding of compound 12 undergoes a "GABA-shift" while the binding of the analog, WE 973 (13),[20] which lacks hynotic activity, is unaffected by GABA.[64] BZ receptor activation has been linked to the modulation of acetylcholine release from rat brain striatal slices.[65] Patch-clamping studies in adrenal chromaffin cells have shown that the dose-response curve for GABA-A receptor-activated chloride channels (which are similar to those in the CNS) has a Hill coefficient consistent with a bimolecular interaction: the GABA-induced currents are potentiated by diazepam.[66]

Peripheral BZ receptors - Since the discovery of PBZRs in the kidney eight years ago, much effort has been expended in elaborating their physiological significance. This is of relevance in the CNS, since such receptors are present in nervous tissue.[67,68] While earlier reports classified PBZRs as "acceptor" sites rather than receptors,[2-4] studies with the agonist Ro 5-4864, and the antagonist PK 11195,[12,69,70] have shown that the receptor may have functional significance. Binding studies have shown PBZRs to be localized in kidney, adrenal cortex, spinal cord, testis, pineal gland and skin. A PBZR can enhance muscimol-dependent phospholipase A-2 activation in C6 glioma cells.[71] Associations between the PBZR and calcium channels have been reported in cardiac tissue,[12,69,70] the GH pituitary cell line,[72] and glial cell cultures.[73] In guinea pig papillary muscle, the effects of the PBZR antagonist PK 11195, the agonist Ro 5-4864, and the calcium agonist Bay K 8644, have been tentatively linked to a model which relates the PBZR to voltage-sensitive calcium channels (VSCCs).[12] However, direct interactions of Ro 5-4864 with VSCCs have also been reported[74] and work in leech neurones has indicated a relationship between a micromolar BZ receptor and calcium channels.[75] Since clonazepam is effective in this system, the BZ receptor may be of the central type. BZ depression of calcium conductance in myenteric neurons has been observed.[76] PK 11195 may be useful for the treatment of angina and cardiac ischemia.[70] Other effects of BZs in non-neural tissues include the antagonism of the actions of platelet aggregation factor[11] (an effect that appears to involve the arachidonic acid pathway),[77] and a chemotactic effect in neutrophils.[78] BZs also inhibit neurite outgrowth in PC12 cells,[79] an action that may be independent of a receptor-mediated event. Endogenous ligands for the PBZR have been reported.[80,81]

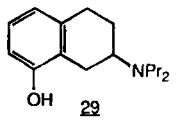
Endogenous ligands - The protein termed DBI (Diazepam Binding Inhibitor) has been isolated and shown to consist of 104 amino acid residues.[10,82] The peptide, which is located in non-GABAergic neurons in rat cerebral cortex, can elicit proconflict responses when administered to rats. Preliminary studies have shown that two fragments of DBI, an octadecapeptide (28 ODN) and an octapeptide (ON), have similar activity to that of the parent moeity.[82] Monoclonal antibodies to 3-hydroxyclonazepam have

been used to identify an endogenous BZ-like factor in mammalian brain.[83] Although clonazepam was used to raise the antibodies, they have limited affinity for the parent drug.[84] They bind Ro 5-4864, diazepam, and flunitrazepam but not Ro 15-1788, compound 7 or the beta-carbolines. While reports related to GABA-modulin continue to appear,[85] this entity has still to fulfil several of the criteria proposed for endogenous ligands.[86]

Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys 28

Opiate and thyrotropin releasing hormone (TRH) receptor interactions - The antagonistic effects of BZs on the antinociceptive actions of cholecystokinin-8 sulfate have been related to effects on CCK-mediated opioid peptide release-effects that may reflect a non-specific calcium antagonist-like action.[87,88] Naloxone can enhance the depressant effects of chlordiazepoxide on mouse spontaneous motor activity,[89] while hydroxymethylbetacarboline can antagonize the respiratory depressant effects of fentanyl.[90] These findings have led to the suggestion that there may be a "cross reactivity" between BZ and opiate receptors. BZs can interact with central TRH receptors[91,92] and chlordiazepoxide is a competitive TRH antagonist in the GH3 pituitary cell line.[93] BZ inhibition of [^{3}H]-methyl-TRH binding shows marked regional specificity in brain tissue, with BZs being much less active in amygdala than in striatum.[94] Furthermore, blockade of central TRH receptors in vivo was found to have no functional correlation, either biochemical or behavioral, in terms of agonist or antagonist activity.[94]

Serotonergic aspects of anxiety - Studies on the buspirone analog TVX Q 7821 (2) have shown that this compound is a selective agonist at serotonin 5-HT_{1A} receptors consistent with previous observations that buspirone had serotonin agonist activity.[95] The prototypic 5-HT_{1A} agonist 8-OHDPAT (29), has also been reported to have anxiolytic activity.[96] Gepirone (3), a second-generation buspirone analog with reduced antidopaminergic activity, is currently undergoing extensive preclinical evaluation;[97] a related compound (4), has been reported.[8] Binding of ^{3}H-buspirone is high in parietal cortex and amygdala (in contrast of that of BZs),[6] providing further evidence that buspirone and similar compounds act as "midbrain modulators".[6] Fluprazine (DU 27716, 30) a compound with serotonergic properties has anxiolytic activity[98] as does the $5HT_2$ antagonist,



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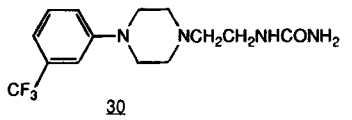

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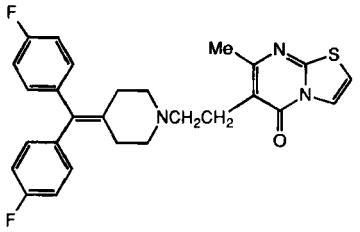
ritanserin (31).[99,100] Compound 32 was reported to be twice as active as chordiazepoxide versus electroshock-induced aggression in mice and was effective clinically.[101] Mechanistically, the compound had weak activity (10^{-6}M) at 5-HT_2 and D-2 receptors, but like other serotonergics did not affect either BZ or GABA binding to the complex. The clinical attractiveness of the buspirone series of antianxiety agents is clouded by the possibility that their intrinsic antidopaminergic activity may result in extrapyramidal symptomatology.[102,103] The reduced incidence of such activity in compounds 2[7] and 3[9] may make these second generation analogs of compound 1 potentially more attractive clinically.



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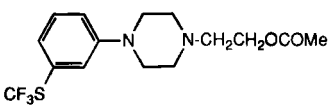

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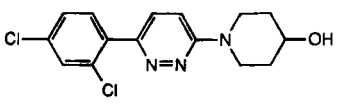
Compound 1 does not induce the serotonin behavioral syndrome[104] and dopaminergic, rather than serotonergic, mechanisms are believed to subserve its activity in a novel acoustic startle model of anxiety.[105] A selective up-regulation of 5-HT_1 receptors in rat frontal cortex has been described with clonazepam, but not with diazepam.[106] This effect has been related to clonazepam's ability to increase brain serotonin levels. Chronic treatment with compound 3 can reduce cortical 5-HT_2 receptors.[9]

Behavioral aspects - With the shift in focus from BZs to serotonergics and other factors, the classical conflict paradigms are proving to be inadequate as preclinical models of anxiety. As a result, acoustic startle,[105] distress vocalization,[107] aggression,[108] brain stimulation reinforcement[109] and defense burying[110] paradigms are undergoing evaluation, as are various neophobic models, including arm maze[111] and open field behavior.[112] This latter paradigm has shown genetic differences in neophobic behavior.[112] An electrically-induced head turning model in rat has been reported to reflect a functional expression of the BZ receptor complex.[113] The evaluation of BZs as antiaggressive agents has been questioned.[114] From behavioral testing of the atypical anxiolytic, compound 30, the term "serenics" has been introduced to describe agents with pronounced antiaggressive, as opposed to anxiolytic, activity. Concurrent biochemical and behavioral studies have led to the identification of an 'anxiolytic locus' in the lateral amygdala.[115] The BZ antagonist, Ro 15-1788, is undergoing evaluation for the treatment of BZ overdose[116] and as a potential analeptic.[117] FG-7142, a beta-carboline with anxiogenic activity, can induce 'learned helplessness'.[118]

Sedative/hypnotics - The aminopyridazine SR 41378 (33) has hypnotic activity.[119] Quazepam has been introduced as a hypnotic. Zopilcone was as effective as temazepam in the treatment of insomnia.[120] The profile of 34 and related analogs is similar to that of methaqualone.[121] 3-Hydroxymethylbetacarboline antagonizes the effects of pentobarbital, suggesting a common locus of action at the BZ receptor.[122] Reports on the hypnotic activity of SC 33963 (35) continue to appear.[123] ZK 93423 (9) is a muscle relaxant.[124]



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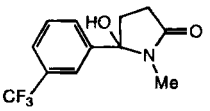

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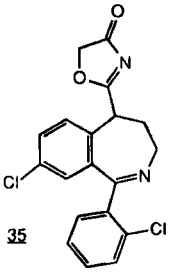

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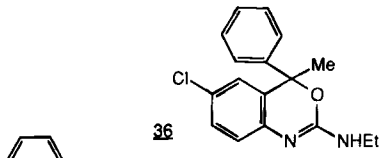

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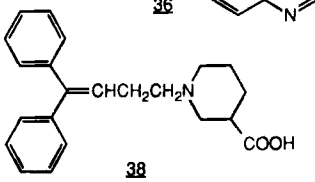

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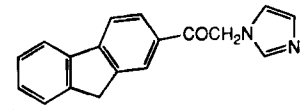

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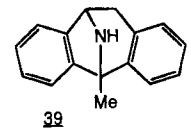

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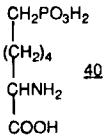
Anticonvulsants - Etifoxine (HOE 36801, 36),[125] ZK 91296 (10)[126] and LY 175644 (37)[127] have been reported as anticonvulsants. Chronic treatment with carbamazepine can up-regulate adenosine A-1 receptors.[128] Adenosine, which may function as an endogenous anticonvulsant, is released following seizure initiation[129,130] and can attenuate, but not prevent postictal spike formation, thereby acting to prevent seizure spread. Prenatal administration of chlordiazepoxide results in a reduced susceptibility to metrazole-induced convulsions in the offspring. This has been correlated with a decrease in the number of BZ receptors in cortex and cerebellum.[131] A deficit in the number of BZ/GABA-A receptor complexes has been reported in substantia nigra and mesencephalic periaqueductal gray matter in the seizure-susceptible gerbil.[132] These decreases, which appear to be genetically predetermined, have been suggested to underlie the etiology of certain types of epilepsy. SKF 89976 (38), a GABA uptake inhibitor, has anticonvulsant activity.[133] Reports on the clinical profile of MK 801 (39) continue to appear.[134]

Novel approaches to anxiety - Antagonists of excitatory amino acids can display similiar pharmacology to compounds which facilitate the actions of inhibitory amino acids. In this context, the N-methyl-D-aspartate antagonist, 2-amino-7-phosphonoheptanoic acid (AP7, 40), has been reported to have anticonflict activity[135] (cf benzodiazepines which facilitate the actions of GABA).[136] Mebicar (41) a novel anxiolytic developed in the USSR is a derivative of allantoin, an oxidation product of uric acid.[137]



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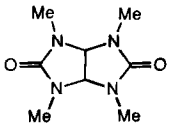

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Chapter 3. Analgesics, Opioids and Opioid Receptors

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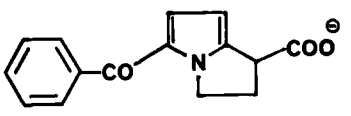
Introduction - Opioid research continues to dominate the search for new analgesic drugs and to provide further insight into the nature and function of endogenous opioid systems. The proceedings of the 1985 International Narcotics Research Conference have been published[1] together with those of a symposium on multiple opioid receptors[2]. The history of opioid research, as reflected in the discovery of buprenorphine, has been assessed.[3] Methods available for evaluating opioid analgesics and antagonists have been reviewed[4] and a collection of articles on agonist-antagonist analgesics has been made available.[5] Reviews have been provided on the mechanisms of pain and opioid induced analgesia,[6] particularly at the spinal level,[7,8] the role of opioid receptor sub-types in relation to antinociception,[9] the significance of opioid-neurotransmitter interactions in analgesia, tolerance and dependence[10] and the role of opioid peptides and antagonists in circulatory shock[11] and in respiratory function.[12] The implications of the pharmacology of quaternary antagonists for their use in future experiments have been discussed[13] and the evidence for stereospecific opioid receptors on excitable cell membranes has been assessed.[14]

CLINICAL HIGHLIGHTS

Non-opioid analgesics - Reviews have appeared on the pharmacology and efficacy of the NSAIDs, tiaprofenic acid[15] and suprofen[16], and on the animal pharmacology of ketorolac tromethamine(1)[17] which has been shown to be as effective (10mg and 30mg, im) as morphine (12mg, im) in the first 6 hours following surgery in man.[18] In patients with cancer pain, diclofenac (75mg, im) was claimed to be significantly better than pentazocine (30mg, im) with fewer side effects[19] and ibuprofen has been used to improve the efficacy of oral narcotics without increasing side effects.[20] Indoprofen (200mg, po) was found to be superior to aspirin (600mg, po) in the relief of dental pain[21] and as effective in post-partum episiotomy pain.[22] Lysine acetylsalicylate has been reported to be an effective analgesic when given by iv infusion for the treatment of post-operative pain.[23,24] A brief review has been provided for emorfazone (2), an antiinflammatory analgesic which increases vascular permeability and inhibits bradykinin release without affecting prostaglandin biosynthesis.[25]



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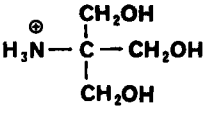

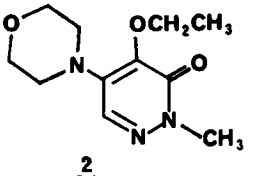

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A pilot study has shown that epidural somatostatin afforded complete analgesia without serious side effects after abdominal surgery in man.[26] Ceruletide has been recommended as a first choice analgesic agent in the treatment of biliary colic pain,[27] whilst infusion of salmon calcitonin (iv) has been shown to be effective in the relief of bone pain in patients

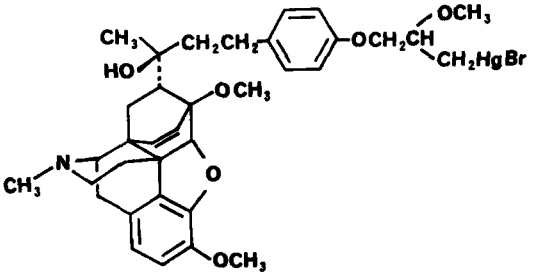
with osteolytic metastases.[28] The methods available for the assessment of pain in healthy volunteers have been reviewed with the emphasis on E.E.G. analysis as a powerful tool for identifying weak analgesic effects[29] and a critical review of pain perception and analgesia as functions of circadian rhythm has been provided.[30]

Opioid analgesics - Newer methods of opioid delivery, including controlled release formulation, sublingual dosing, intravenous infusion and spinal administration, have been reviewed.[31] The strategy of patient-controlled analgesia has been discussed[32] and its application illustrated by morphine infusion (iv) in post-operative pain relief.[33] Epidural injections of morphine administered "on demand", either alone or as a supplement to an epidural infusion, gave good post-operative analgesia with a low incidence of respiratory depression after a variety of surgical procedures.[34,35] Significantly less nicomorphine was needed for adequate post-operative analgesia when given by epidural, rather than im, injection.[36] Preliminary studies showed that dynorphin A (Dyn A) and human β-endorphin (β_H-EP) reduced intractable pain without side effects, when administered intrathecally.[37] In double-blind studies, ciramadol (60mg, im) compared favorably with morphine (10mg, im) as a post-operative analgesic,[38] while dezocine appeared slightly more potent than morphine on a mg basis and gave a more rapid onset of analgesia.[39] The pain relief provided by viminol (5mg, iv) was not significantly different from that obtained with pentazocine (30mg, iv) post-operatively, though the incidence and severity of side effects was less.[40] Buprenorphine has been shown to be satisfactory for major surgery as part of a balanced anaesthetic technique.[41] It has been suggested that the differences in analgesic profiles of buprenorphine and fentanyl in man can be explained in terms of their pharmacodynamic properties as observed in in vitro receptor binding assays.[42] Reviews have appeared on the animal pharmacology and clinical evaluation of sufentanil,[43] naltrexone[44] and meptazinol.[45] In recent studies meptazinol (175mg, im) was equivalent to morphine (10mg, im) in the relief of severe post-operative pain[46] while 100mg (im) was equivalent to, or better than, morphine (15mg, im) in the treatment of pain due to traumatic injury.[47] The cholinergic component of meptazinol has been ascribed to its inhibitory effect on cholinesterases.[48]

OPIOID RECEPTOR STUDIES

Receptor isolation - Progress has been reported towards the purification of sufficient binding-site material for sequencing, using techniques involving detergent solubilization, affinity chromatography and gel electrophoresis of rat brain membranes. When [D-Ala^2-Leu^5]enkephalin (DALE) was used as the affinity ligand,[49] a 450-fold purification of binding sites with molecular weight 62,000 was achieved.[50] Studies with hybromet (3), an opioid claimed to have high affinity for μ-receptors,[51] afforded a 500-fold enrichment of a single class of high affinity sites comprising three sub-units of molecular weights 94,000, 44,000 and 35,000, respectively.[52] A 3,200-fold purification of an opioid binding fraction was obtained with 6-succinylmorphine as the ligand and conditions which allowed co-elution of acidic lipids. The purified material exhibited high affinity, stereospecific, Na^+-dependent binding of a variety of opioids, with the same rank order of potency observed for membrane bound μ-receptors.[53] The potent δ-selective acylating agent 4 has been used to purify an opioid receptor sub-unit from NG108-15 neuroblastoma-glioma hybrid cells by 30,000-fold, affording a homogeneous protein with molecular weight 58,000, which showed a strong tendency to dimerise.[54] In another study, irreversible cross-linking with [^{127}I]-β_H EP, followed by gel electrophoresis,

revealed major bands with molecular weight 65,000 from rat thalamus and 53,000 from NG108-15 cell membranes, considered to be μ- and δ-sites, respectively.[55] β-Naltrexyl-6-ethylenediamine was used as the affinity ligand for the isolation of μ-sites from bovine striatum, affording 60-75,000 fold purification of a protein with molecular weight 65,000.[56]



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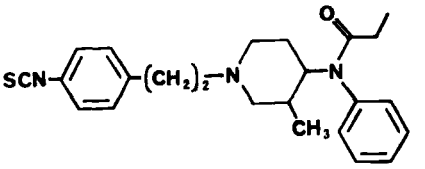


4

Receptor classification and pharmacology - The use of ligands with limited selectivity (<100-fold) for one opioid receptor type, in tissues containing low concentrations of that site, may produce effects mediated through other opioid receptors.[57] Sophisticated techniques, often using non-physiological conditions to maximize the interaction of compounds with their primary recognition sites, continue to be reported. The results of such studies should be interpreted with caution.

The differential ontogeny of the commonly accepted μ-, δ- and k-receptor types has been demonstrated in the rat brain.[58] Species differences have been shown in the concentrations and distributions of opioid binding sites[59] and differential laminar distribution has been found in rat and guinea pig spinal cord.[60] Computer analysis of the binding of tritiated dihydromorphine (DHM), DALE and ethylketocyclazocine (EKC) to mouse brain, identified a fourth site which bound EKC with high affinity but which did not bind naloxone.[61] A preliminary report has appeared of an intact brain cell assay in which [^{3}H]-naloxone binds in a saturable, concentration dependent, stereospecific manner, but a high level of non-specific binding occurs with [^{3}H]-DHM.[62]

μ-Receptors - β-Funaltrexamine (β-FNA), an irreversible antagonist with μ-selectivity on the guinea pig ileum (GPI),[63] has been used to identify a subset of GPI binding sites, termed μ′, which interact only with opioid peptide agonists yet can be blocked by naloxone.[64] Evidence has been presented which suggests that despite their relative lack of selectivity *in vitro*, [^{3}H]-diprenorphine[65] and [^{3}H]-etorphine[66] bind predominantly to μ-receptors *in vivo*. Compatible results were obtained *in vivo* and *ex vivo* with a series of oripavines but considerable differences were noted from corresponding *in vitro* data.[67] These studies suggest that direct comparison between receptor binding and pharmacological response must rely on binding data obtained in intact, live tissue.[66] Binding studies with dermorphin have suggested that it interacts preferentially with μ-receptors.[68] Chronic administration caused tolerance to the analgesia produced in the rat (icv) and led to dependence.[69]

δ-Receptors -Further characterization of the δ-receptor on the hamster vas deferens has been reported.[70] The mechanism by which δ-receptors modulate adenylate cyclase in cultured NG108-15 cells continues to be explored.[71-73] Studies with [D-Ala^2-D-Leu^5]enkephalin (DADLE) have indicated that multiple affinity states of the receptor exist in intact cells under coupling conditions[74] and this has been confirmed for a number

of opioids by computer analysis of the binding isotherms.[75] A preliminary report has appeared suggesting the presence of [D-Ala^2-Met^5]enkephalinamide binding sites which regulate adenylate cyclase activity in human neuroblastoma cells.[76]

μ- and δ-Receptors - The concept that μ- and δ-receptors co-exist in a single receptor complex has been suggested from computer curve-fitting techniques applied to *in vitro* data,[77,78] and from a rat flurothyl seizure model *in vivo*.[79] However, kinetic analysis of the effects of ions and nucleotides on opioid binding to rat brain membranes was said to support the existence of two independent μ- and δ-binding sites.[80]

k-Receptors - The profile of k-agonists in the rabbit vas deferens (which does not respond to μ- or δ-agonists) implies that this tissue has a high intrinsic activity requirement.[81] Pretreatment of rat brain membranes with μ- and δ-selective site-directed acylating agents afforded a preparation highly enriched with k-binding sites.[82] Data have been presented which indicate that [^{3}H]-EKC binding (μ- and δ-suppressed) to rat brain sites is differentially affected by Na^+ and is GTP sensitive, though less so than the binding of other opioids.[83] Binding studies with a variety of tritiated k-selective ligands provide evidence that k-receptors are heterogenous in guinea-pig brain,[84] rat brain[85] and bovine adrenal-medula.[86] The finding that Dyn A-related peptides did not generalize to the EKC-discriminative stimulus in rats was attributed to the possible existence of k-receptor subtypes.[87]

The putative endogenous k-ligand, Dyn A,[88] is a more potent analgesic in mice when given intrathecally as opposed to intracerebroventricularly (icv).[89] In rats the opioid antinociceptive effects of Dyn A at the level of the spinal cord did not appear to be μ-related.[90] It has been proposed that the analgesic and motor effects observed in this model may be k-mediated.[90,91] In addition, an increase in spinal immunoreactive Dyn A has been demonstrated in response to chronic arthritic pain in the rat.[92] On DRG neurons in cell culture dynorphin-gene products appeared to act at opioid receptors coupled to voltage dependent calcium channels which were neither μ- nor δ-sites.[93]

ε-Receptors - Further studies have been reported to characterize the binding of β_H-EP to the putative ε-receptor in the rat vas deferens (rvd).[94] In rat brain, the distribution of β_H-EP immunoreactivity was found to be similar to that of benzomorphan binding sites but different from μ-, δ- and k-sites.[95] The correlation ($r = 0.96$) observed between the effects of pro-enkephalin A-derived peptides (including β_H-EP) on the rvd and analgesia in the mouse tail-flick model (icv) was tentatively attributed to ε-receptor occupancy, though the paucity of biochemical information about the ε-receptor did not allow more definite conclusions.[96]

ENDOGENOUS OPIOIDS

Physiology and pharmacology - Evidence for the involvement of opioid peptides in the immune response to stress and in tumor growth has been reviewed.[97-99] Footshock stress (thought to release endogenous opioids) has been shown to suppress immune function and decrease resistance to tumor challenge in rats - effects mimicked by morphine administration.[100] The origins of the diversity in opioid peptides have been reviewed[101] and specific studies in the adrenal-pituitary axis have been reported.[102] Reviews have been provided on endorphins and exercise,[103] the involvement of endorphin-related peptides in non-opioid effects associated with stress and the endocrine system,[104] and the endocrinology of opioids.[105]

New peptides - The post-translational processing of opioid peptide precursors, and the possible function of the products, has been briefly reviewed with the emphasis on leumorphin.[106] Amidorphin, isolated from bovine adrenal-medulla, was shown to correspond to residues 104-129 of bovine proenkephalin A, amidated at the C-terminus.[107] Immunoreactive metorphamide, the only other C-amidated endogenous opioid known, has been found in human adrenal extracts.[108] Two peptides which have a common C-terminal tetrapeptide sequence and cross react with an antiserum raised against FMRF-NH_2 (H-Phe-Met-Arg-Phe-NH_2) have been isolated from bovine brain.[109] Both decrease tail-flick latency in the rat (icv) and the smaller octapeptide, H-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2, also attenuates morphine analgesia in this model.[109]

Peptide antagonists - β_H-EP-(1-27) and Tyr-MIF-1 (H-Tyr-Pro-Leu-Gly-NH_2) have been shown to block the analgesic effects of opioids in the mouse, implying that these endogenous ligands might act physiologically as antagonists.[110,111] In addition, MIF-1 significantly increased pain scores in human volunteers receiving morphine.[112] Studies with proglumide (4-benzoylamino-5-dipropylamino-5-oxopentanoic acid), a cholecystokinin (CCK) receptor antagonist, demonstrated that it potentiates the analgesic effect of morphine,[113] β-endorphin[114] or environmentally-induced release of endogenous opioids.[115] Proglumide attenuated tolerance to the analgesic effects of morphine in the rat[116,117] and significantly potentiated morphine analgesia in human volunteers.[118] These findings have been interpreted to support the hypothesis that CCK-8 may function physiologically to antagonize the effects of endogenous opioids.

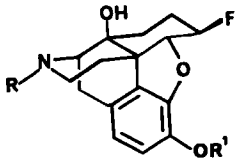
Enzyme inhibition - The concept of modifying peptidergic neurotransmission by inhibition of enkephalin-degrading enzymes has been reviewed.[119] Studies with rigid analogues and the enantiomers of thiorphan, obtained by asymmetric synthesis,[120] have shown no correlation between enkephalinase A inhibition and analgesic potency.[121] The S-isomer of thiorphan appeared to be responsible for angiotensin converting enzyme inhibition and, while both R- and S-forms were equally effective against enkephalinase *in vitro* and *in vivo*, analgesic activity resided mainly in the R- isomer.[122] The synthesis and analgesic properties of a series of bidentate ligands has been described. Kelatorphan (N-[3(R)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]-L-alanine) was the most potent and inhibited three different metallopeptidases, as did other analogues.[123] Preliminary evidence has been published for a new receptor-associated aminopeptidase which degraded Met^5-enkephalin and co-purified with opioid receptors.[124]

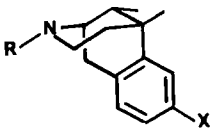
Morphine - The isolation and characterization of non-peptide opioids from bovine brain and adrenal samples[125] and from toad, rat and rabbit skin has been reported.[126] The material from toad skin, and one of the four substances purified to homogeneity from bovine extracts, were identical with morphine by immunological, pharmacological and physicochemical criteria. Biosynthetic evidence would be necessary to establish that morphine is an endogenous ligand *in vivo*.[125,127]

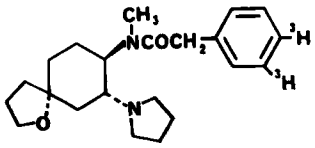
OPIOID RECEPTOR PROBES, NOVEL COMPOUNDS AND ANALGESICS

Opioid receptor probes - The available affinity labels have been reviewed with emphasis on the utility of irreversible electrophilic antagonists such as β-FNA.[128] In primates, β-FNA pretreatment selectively antagonized the behavioral effects of acute morphine but not those elicited by EKC.[129] However, in certain other experimental paradigms, β-FNA afforded long-lasting blockade of μ- and δ-receptors both *in vitro*[63] and *in vivo*.[130] The

syntheses of various ^{11}C-labeled ligands suitable for positron emission tomography have been reported, including lofentanil,[65] carfentanil[131] and diprenorphine.[132,133] It has been suggested that incorporation of ^{18}F into 6-deoxy-6β-fluorooxymorphone (5) and 6-deoxy-6β-fluoronaltrexone (6),[134] or (+)-N-(3-fluoropropyl)normetazocine (8),[135] could provide ligands with longer half lives for this technique: the synthesis of [^{18}F]-3-acetylcyclofoxy (7) has been reported.[136]







5 R = CH_3, R^1 = H

6 R = CH_2-cyclopropyl, R^1 = H

7 R = CH_2-cyclopropyl, R^1 = $COCH_3$

8 R = $(CH_2)_3F$, X = OH

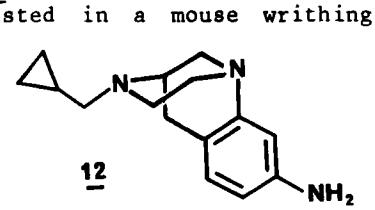
9 R = CH_3, X = $SCOC_6H_5$

10 R = CH_3, X = OH

11

The synthesis and evaluation of tritiated opioids which are selective for δ- and k-receptors has been reported. Studies with [^3H]-[D-Pen^{2,5}]-enkephalin ([^3H]-DPDPE) in NG108-15 cells have confirmed that it is a potent and selective δ-ligand.[137] It has been suggested that [^3H]-DPDPE can be used to examine δ-receptors in regions containing a high proportion of μ-sites[138] and it has been utilised to demonstrate a unique distribution of δ-sites in rat brain.[139] [^3H]-[D-Thr^2,Leu^5]enkephalyl-Thr^6 has been proposed as an alternative ligand by other workers who have claimed that [^3H]-DPDPE exhibits low δ-affinity in NG108-15 cells, thereby inducing high experimental errors and cancelling the benefits of its δ-selectivity.[140] [^3H]-Tifluadom was shown to be more specific for the k-receptor than [^3H]-bremazocine or [^3H]-EKC[141] but none of these ligands was said to be selective enough for meaningful studies.[142] An analogue of U50488H, [^3H]-U69593 (11), has recently been shown to bind with high affinity to guinea-pig, mouse and rat brain membranes, with a high preference for k- over μ-sites.[143]

Novel compounds - The (+)-9-amino analogue 12 was the most potent of a series of 1,5-methano-1,4-benzodiazocines tested in a mouse writhing assay.[144] 8-Benzoylthio-2,6-methanobenzazocine (9) was half as active as metazocine (10) and less toxic in the mouse.[145] The (-)-acetylmorphinan derivative 13 was orally active and comparable in potency to morphine in analgesic tests and naloxone binding, yet with little or no dependence liability in morphine dependent rats.[146]



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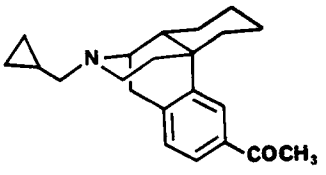

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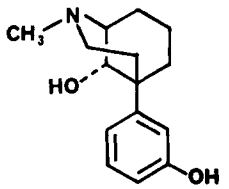

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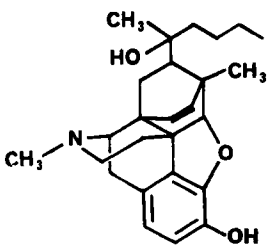
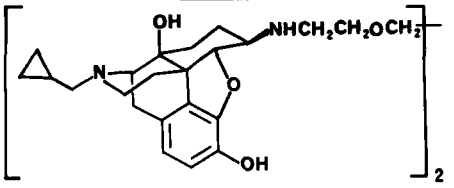


15

Analgesic activity equivalent to morphine has been reported for the racemic 9α-hydroxyphenylmorphinan 14 whereas the corresponding 9β-isomer

was virtually inactive.[147] The (R)-Diels-Alder adduct 15, derived from 6-deoxythebaine, was a potent analgesic in a rat tail-flick model (2200 x morphine, sc).[148] Selective k-antagonist activity has been claimed for TENA (16), a dimeric β-naltrexamine analogue which is 27-fold more effective versus U50488 than morphine in vitro.[149] Electron acceptor properties of the aromatic ring and amine functions, and conformational energy constraints, appear to play a significant role in determining μ-binding affinity in a series of trans-N-[2-(methylamino)cyclohexyl]benzamides (17).[150]



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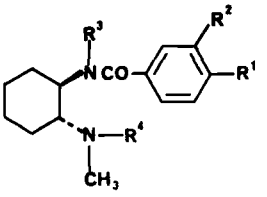

17

The rigid 13-membered ring compound, H-Tyr-D-Orn-Phe-Asp-NH_2, has been claimed to be one of the most selective μ-ligands known, while the more flexible peptide H-Tyr-*D*-Lys-Phe-Glu-NH_2 (15-membered ring) showed no preference for μ- over δ- receptors in binding studies.[151] In addition, efficient binding and μ-selectivity has been demonstrated for the somatostatin analogue, H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-ThrNH_2 (CTP) which contains a 20-membered ring.[152] Studies with modified analogues of dermorphin (18) have shown that the guanidine 19 is more potent than the parent peptide in producing both analgesia and akinesia in rats,[153] while the partially retro-inverso tetrapeptide 20 is equivalent to dermorphin in the mouse tail-flick test (icv) and five times more potent in vitro.[154] The pentapeptide dimer 21 was half as potent as DALE-amide in a rat analgesic assay (icv),[155] whereas the tripeptide dimer 22 approached morphiceptin in terms of potency and μ-selectivity in rat brain and NG108-15 cell binding assays.[156]

X—Tyr-D-Ala—Phe—Y

18 X = H, Y = Gly—Tyr—Pro—Ser—NH_2

19 X =  , Y = Gly—Tyr—Pro—Ser—NH_2

20 X = H, Y = $NHCH_2NHCO$— 

(Tyr-D-Ala—Gly—X—CH_2)$_2$

21 X = Phe—Leu—NH

22 X = NH

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Chapter 4. Cognitive Disorders

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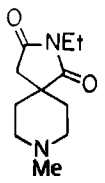
Introduction - Memory dysfunctions have been reviewed from preclinical and clinical perspectives.[1-4] No effective therapy exists for primary degenerative dementia (PDD, Alzheimer's disease), mild (or minimal) memory impairment (benign senescent forgetfulness), or for multi-infarct dementia (MID). However, a better understanding of these disorders and their prevalence, of the biological changes accompanying cognitive impairment and decline, and the development of animal models based on increasing knowledge of affected brain systems, gives hope that new treatments based upon rational approaches will soon be available.

PDD remains the focus of most clinical research on cognitive disorders, but the pseudo-dementias, Korsakoff's syndrome, geriatric depression, and attentional deficit disorder are beginning to receive appropriate attention. Development of effective therapy for PDD is hindered by unknown (perhaps multiple) etiologies, the absence of efficacious agents to validate preclinical models, and the lack of early, reliable diagnostic methods. Possible causes of PDD include genetic predisposition, altered proteins (e.g. amyloid), infectious agents, toxins, blood-flow disorders, neurotransmitter abnormalities, and multiple factors.[5,6]

Cholinergic system - The cholinergic hypothesis[7] remains a viable explanation for memory deficits in PDD, but it is unlikely to account for the entire range of biochemical, pathological, anatomical, and pharmacological deficits in dementia.[8-11]

Clinical studies in PDD with muscarinic agonists (e.g. RS-86, 1 and intracranially-infused bethanecol, 2),[12,13] cholinesterase inhibitors (e.g. physostigmine, 3),[14,15] and cholinergic releasing agents (e.g. 4-aminopyridine)[16] showed significant, albeit weak, activity. Cholinergic precursors such as choline (Ch) were inactive.[17] Minaprine (4), which facilitates serotonergic and dopaminergic neurotransmission, and also has "procholinergic" activity, may be more effective in MID than in PDD.[18,19]



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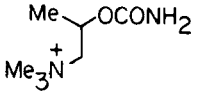

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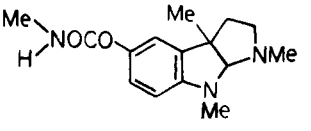

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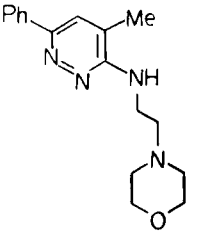
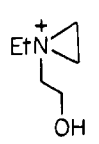


4

Scopolamine challenges represent a potential diagnostic tool for PDD. PDD patients may be supersensitive to muscarinic antagonists because of their underlying cholinergic deficits.[20] In vivo imaging of muscarinic receptors in a PDD patient, using single photon emission computed tomography, showed impaired binding of ^{123}I-labeled quinuclidinyl benzilate.[21]

Animal models for cognitive disorders remain an area of intense research, but an appropriate model for PDD does not exist. Physostigmine and cholinergic ventral forebrain grafts into the neocortex alleviate memory deficits caused by ibotenic acid lesions of the nucleus basalis of Meynert in rats as measured by passive avoidance.[22,23] Ibotenic acid lesions of the basal forebrain impair serial reversal learning in marmosets with low doses of scopolamine that do not affect controls.[24] Ibotenic acid lesions of the nucleus basalis disrupt, and physostigmine enhances, performance on delayed matching in rats.[25,26] In the rat, functional and neurochemical cortical cholinergic impairment results from lesioning the nucleus basalis magnocellularis with kainic or quinolinic acid.[27] Cholinergic neurons of the basal nucleus degenerate following kainic or N-methyl-D-aspartic acid application to rat cerebral cortex.[28] Aziridinium analogues of choline (AF64A, 5)[29] and oxotremorine (BM130A, 6)[30] may be useful in developing animal models of cholinergic hypofunction. AF64A causes long-lasting, marked reductions in acetylcholine (AcCh) levels and release, in high affinity Ch uptake, and in Ch acetyltransferase (ChAcT) activity, with no effect on muscarinic receptor binding. Use of the choline false precursor, N-aminodeanol (7), was proposed for development of animal models of cholinergic hypofunction.[31] Inbred strains of rodents may ultimately be useful in establishing new models of cognition sensitive to cholinergic agents.[32]



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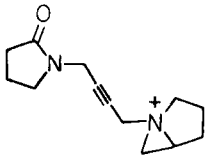

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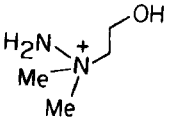
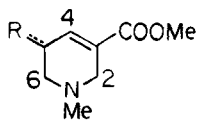
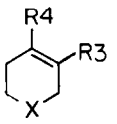


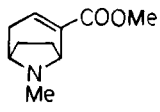
7

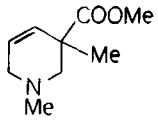
Muscarinic receptors were reviewed.[33,34] Several studies reported functional and biochemical bases for multiple muscarinic receptors,[35,36] but classification problems remain.[37,38] Reductions in M2 receptor number and in ChAcT activity are observed in cortical samples from PDD patients and from rats cholinergically denervated with ibotenic acid. M1 receptors are unchanged. M2 receptors may regulate AcCh release, with M1 receptors facilitating postsynaptic cellular excitation.[39] Theoretical methods were used to describe cholinergic neurotransmitter receptor interactions.[40,41]

Analogues of arecoline (8) were evaluated in smooth muscle and cardiac tissue preparations: examples included the dihydro derivative, esters of arecaidine and isoarecaidine 11 and 12, the thiane 13, and the rigid analogue 14.[42] The deconjugated and 5-substituted derivatives 15, 9, and 10 were studied as γ-aminobutyric acid uptake inhibitors, but cholinergic activity was not investigated.[43] Combinations of agonists (e.g. arecoline plus oxotremorine) display supra-additive enhancement of memory in a mouse T-maze active avoidance paradigm.[44]









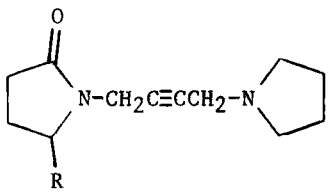
8 R=H
9 R=$Me_2C(OH)$-
10 R=Me_2C=

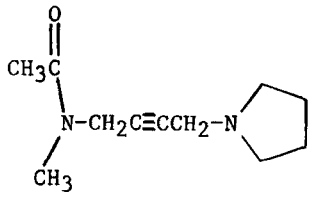
11 R3=COOH,R4=H,X=NMe
12 R3=H,R4=COOH,X=NMe
13 R3=COOMe,R4=H,X=SMe+

14

15

The oxotremorine (16) analogue 17 stimulates inositol phospholipid turnover.[45-48] BM-5 (18) is a presynaptic antagonist and a postsynaptic agonist.[49]

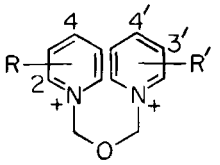




16 R = H
17 R = CH_3

18

The bisquaternary pyridinium oximes 19-23 are allosteric inhibitors of the muscarinic receptor.[50] No allosteric activators have yet been reported.

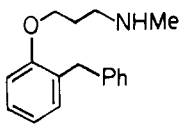


	R	R'
19	2-CH=NOH	3'-COPh
20	2-CH=NOH	3'-$COcC_6H_{11}$
21	2-CH=NOH	3'-COiBu
22	2-CH=NOH	4'-$CONH_2$
23	4-CH=NOH	4'-CH=NOH

Most research on cognition and the cholinergic system has focused on muscarinic receptors. However, nicotinic receptors are increasingly of interest as evidence accumulates for changes in both muscarinic and nicotinic receptors in PDD.[51] Nicotine affects attention, memory, and rapid information processing in humans,[52,53] and learning and memory in mice as measured by passive avoidance.[54] It also stimulates AcCh release,[55] and, in humans, counteracts depressed performance caused by scopolamine.[56]

The cholinergic system interacts with several other neurochemical systems.[57,58] Examples include serotonin,[59] amino acids,[60] adenosine,[61] adenylate cyclase, and phosphodiesterase (PDE).[62] The potential exists, therefore, for many classes of agents to alleviate the cholinergic deficit in PDD. Nerve growth factor (NGF) may be involved in the normal functioning of forebrain cholinergic neurons as well as in neurodegenerative disorders. The biochemical and neurochemical effects of NGF on ChAcT activity[63,64] and in adult rats with fimbria lesions were described.[65] Bifemelane (MCI-2016, 24), reported to be a

"cholinergic potentiator," ameliorates age-related decreases in AcCh release and exerts a protective effect on cerebral ischemia in gerbils.[66] Chronic administration of the thiamine derivative salbutiamine (25) improves long-term memory in mice, possibly by a cholinergic mechanism.[67]



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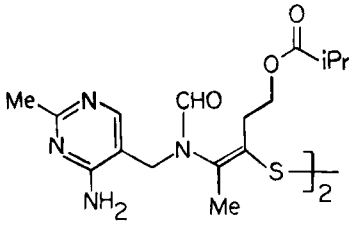

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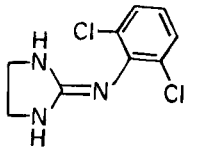
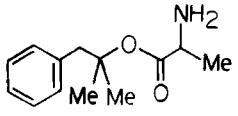


26

Biogenic amines - Clonidine (26) ameliorates cognitive deficits in aged monkeys on a delayed response task. The site of action may be the prefrontal cortex where catecholaminergic neurites are found in senile plaques.[68,69] However, clonidine impaired paired-associate learning but had no effect on short or long-term memory in normal humans.[70]

Alaproclate (27), a specific inhibitor of neuronal serotonin (5HT) reuptake, may have a positive effect on emotional functions in PDD;[71] others report little effect in MID and PDD.[72] Zimeldine (28), another 5HT reuptake blocker, has no effect on memory or reaction time in PDD.[73] The hallucinogenic amphetamine derivative MDA (29), which selectively destroys serotonergic nerve terminals, may aid in developing animal models of serotonergic deficit.[74]



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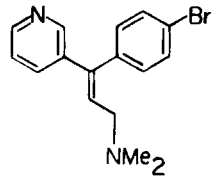

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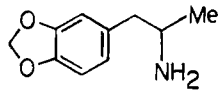
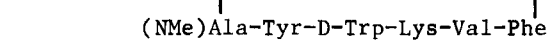


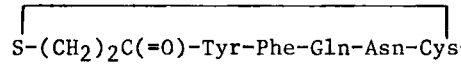
29

Neuropeptides - Somatostatin-like immunoreactivity (SLI) is reduced in parietal but not frontal cortex, and in the hippocampus and putamen in PDD.[75,76] Decreases in somatostatin receptor number correlate with SLI decline, suggesting either degeneration of postsynaptic neurons, or increased somatostatin release leading to postsynaptic down-regulation.[77] SLI is present in neuritic plaques,[78,79] and a subclass of somatostatin-containing neurons selectively affected in PDD contains neuronal tangles.[80] Low cerebrospinal fluid (CSF) levels of somatostatin were found in PDD patients.[81] The somatostatin analogue L-363,586 (30) did not improve memory impairment in PDD patients, but the absence of side effects suggests that higher doses should be tried.[82] Evaluation of linear and cyclic forms of somatostatin, as well as certain fragments (somatostatin 3-6, 7-10), indicates that the entire peptide sequence is required for prevention of electroconvulsive shock (ECS) induced retrograde amnesia in rats.[83]

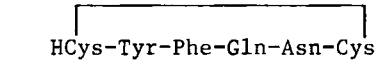


30

Vasopressin (VP) enhances memory though probably not by central mechanisms. Its behavioral effects may involve peripheral reinforcement and central modulation of arousal.[84] The VP analogue DDAVP (31) facilitated memory in males,[85] but not in females.[86] DDAVP did not improve the memory impairment of chronic schizophrenics,[87] nor was it effective in reversing ethanol-induced deficits in memory or cognitive function in normal subjects.[88] Another VP analogue, DGAVP (32), was not effective in PDD or Korsakoff's Disease.[89] In a separate study, DGAVP produced slight improvement in list learning of low imagery words.[90]



$S\text{-}(CH_2)_2C(=O)\text{-}Tyr\text{-}Phe\text{-}Gln\text{-}Asn\text{-}Cys\text{-}Pro\text{-}Arg\text{-}GlyNH_2$ 31

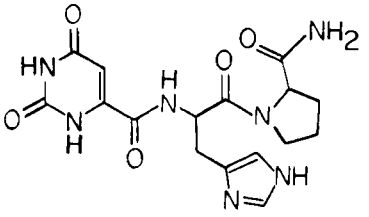


HCys-Tyr-Phe-Gln-Asn-Cys-Pro-ArgOH 32 HMet(S=O)-Gln-His-Phe-Lys-Phe 33

ORG 2766 (33), a synthetic analogue of ACTH 4-9, was well tolerated but failed to produce significant effects on cognitive function in PDD.[91] ORG 2766 was not beneficial on impaired performance related to working overnight or disturbed sleep.[92] It did not reduce severity of depression or anxiety,[93] or improve learning, consolidation, or retrieval of memorized material in the elderly, although a higher degree of sustained attention was noticed during drug treatment.[94] CSF levels of ACTH are low in PDD.[95]

β-Endorphin produces a retrograde amnesia in animals which is blocked by opiate antagonists (e.g. naloxone) and by cholinergic agents (e.g. oxotremorine, physostigmine).[96,97] Dynorphin 1-13 does not cause retrograde amnesia in rats.[98] CSF levels of β -endorphin are low in PDD and MID, but levels of β-lipotropin are unchanged.[99] Naloxone did not improve impaired cognition in PDD,[100] or in depressed patients after ECS therapy.[101]

The therapeutic potential of thyrotropin releasing hormone (TRH) and its analogues in treating PDD and amyotrophic lateral sclerosis was reviewed.[102] Two stable TRH analogues were evaluated against experimentally-induced spinal trauma in cats: CG-3509 (34) improved neurologic recovery but MK-771 (35) did not.[103]



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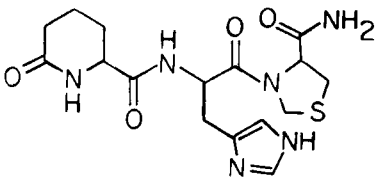
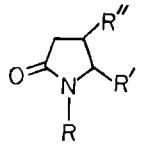


35

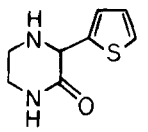
CCK-8 (cholecystokinin-8; sulfated and nonsulfated) was marginally active in preventing ECS-induced amnesia in rats (i.c.v.), and may indirectly influence memory.[104] CSF levels of vasoactive intestinal polypeptide are unchanged in PDD, but reduced in MID.[105] Neuropeptide Y (NPY) may inhibit memory retention in PDD.[106] NPY-like immunoreactivity is found in PDD neuritic plaques.[107] Neurotensin immunoreactivity is unaltered in several brain regions in PDD, but is increased in the frontal and temporal cortex of Down's patients. The latter changes appear related to the presence of plaques and tangles.[108] Angiotensin-

II facilitates learning and retention in active avoidance paradigms in shuttle-box-trained rats when administered (i.c.v.) before or after training. Saralasin, while having no effects of its own, blocked the effects of angiotensin-II on learning.[109] The peptide HPhe-Ile-Tyr-Ser-Tyr-LysOH (36) reverses ECS-induced amnesia and facilitates T-discrimination task learning in rats.[110]

Nootropics and other agents - Most compounds described as nootropics are pyrrolidone derivatives, the first example being piracetam (37). Piracetam-treated dyslexic boys were faster readers, but no effects were observed on reading accuracy or comprehension. Piracetam improved auditory short-term memory in individuals with relatively poor memory,[111] produced clinical improvement in some adult males with alcoholic psychosis,[112] and increased cerebral blood flow and somatosensory evoked potentials in hypotensive cats.[113] Peak serum concentrations are reached within one hour, and little metabolism occurs.[114] Specific combinations of piracetam and Ch enhanced habituation of exploratory activity in mice, but neither drug alone had an effect.[115] Piracetam-Ch combinations affect hippocampal rhythmic slow activity in rabbits.[116] Piracetam potentiates the anticonvulsant effects of carbamazepine.[117]



37 R = CH_2CONH_2, R' = R" = H
38 R = C(CN)=CHNMe$_2$, R' = R" = H
39 R = CH_2CONH_2, R' = H, R" = OH
40 R = $CH_2CONH(CH_2)_2N(iPr)_2$, R' = R" = H
41 R = 4-MeOPhCO, R' = R" = H
42 R = 4-MeOPhCO, R' = $(CH_2)_2COOH$, R" = H



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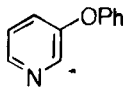

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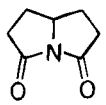

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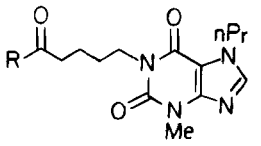
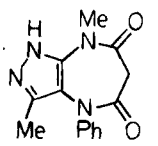
Structure activity relationships were proposed for piracetam analogues that affect spontaneous locomotor activity, ECS-induced retrograde amnesia, and survival under hypoxic conditions.[118] Compound 38 is twice as active as piracetam in protecting mice against acute hypoxia.[119] Oxiracetam (ISF-2522, 39) was more effective than piracetam in improving neurological and somatic symptoms associated with chronic cerebrovascular insufficiency in elderly patients, possibly via platelet aggregation inhibition.[120] Oxiracetam is excreted unchanged (>90%) in urine after oral administration.[121] The synthesis and activities of a series of analogues of pramiracetam (CI-879, 40) were described.[122] Pramiracetam shows a "therapeutic window" in terms of effects on learned behavior, gross EEG activity of the hippocampus and frontal cortex, and firing rate of single hippocampal neurons.[123] Aniracetam (Ro-13-5057, 41) improved the accuracy of pigeons and monkeys on variable-delay matching-to-sample and antagonized scopolamine-induced impairment in monkeys.[124] The activity of tenilsetam (CAS-997, 43) in animal models of cognition was reviewed.[125] Beneficial effects observed with CAS-997 may reflect positive actions on metabolic and cholinergic functions.[126] The chemistry and pharmacology of CI-844 (44),[127] rolziracetam (CI-911, 45),[128] and CI-933 (42)[129] were briefly reviewed.

Pentoxifylline (46), a PDE inhibitor, reverses age-related spatial memory deficits.[130] Propentofylline (HWA 285, 47) reverses cycloheximide-induced amnesia in mice,[131] and affects rat cerebral metabolism[132] and human psychomotor performance, mood, and memory.[133] Hoe 175 (48) enhances memory in mice.[134] Pyritinol (49) facilitates retention of a learned task in poor learners only.[135] Idebenone (CV-2619, 50) protects against cerebral ischemia-induced amnesia in rats.[136] Sulfoxazine (Y-8894, 51) protected mice against ECS-induced amnesia and hypoxia but had no effect on learning and memory.[137] Buflomedil (52) was well tolerated in demented patients, and some improvement in cognitive function was noted.[138]



46 R=Me
47 R=Et



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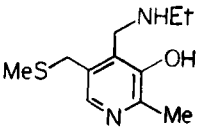

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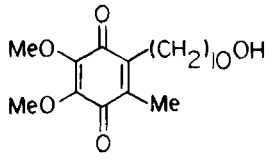

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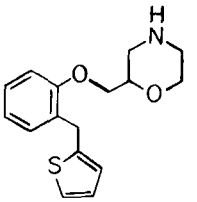

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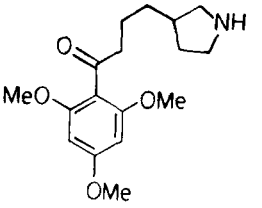

52

While preclinical and preliminary clinical studies have often been encouraging with the newer cognition activators, well-designed, double-blind, placebo-controlled trials have thus far failed to demonstrate unequivocal drug effects.

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Chapter 5. Drugs Acting at Central 5-Hydroxytryptamine Receptors

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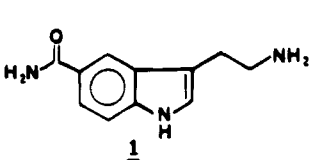
Introduction - Since the last review on central 5-hydroxytryptamine (5-HT, serotonin) receptors appeared in this series, major advances have been made in the definition of the functional consequences of the stimulation and blockade of these multiple receptor sites in the CNS.[1] To a large degree, these advances have been made possible by the availability of drugs, both agonists and antagonists, with a degree of selectivity and activity at the various central 5-HT receptor subtypes. The purpose of this review is to identify the key drugs which have led to these advances and to give an indication of their potency and selectivity at central 5-HT receptors. Central 5-HT_1 receptors have been the subject of several recent reviews and monographs.[1-9] Important advances have also been made in the identification of compounds with effects at peripheral 5-HT receptors and the reader is referred to a series of reviews on this subject.[9]

Central 5-HT receptor classification - On the basis of radioligand binding studies, central 5-HT recognition sites were originally classified into two subtypes, designated 5-HT_1 and 5-HT_2.[10] It is now generally accepted that the 5-HT_2 recognition site, defined on the basis of high affinity binding of [^{3}H]-spiperone or [^{3}H]-ketanserin, has many functional correlates in the CNS and the periphery.[11] Correlates of the 5-HT_1 recognition site, which is labeled by [^{3}H]-5-HT, have proven more difficult to determine and indeed the relationship of these binding sites to functional receptors has been questioned.[1,11] Over the last few years, however, it has become increasingly evident that the original definition of the 5-HT_1 site as a single homogeneous entity was an oversimplification and that at least three subtypes may exist. These have been designated 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C}.[12,13] Extensive autoradiographic studies of the regional distribution of central 5-HT recognition sites have been published.[14,15]

5-HT_1 Recognition sites - A number of compounds shows selective affinity for 5-HT_1 as compared to 5-HT_2 recognition sites. These include the putative agonists, 5-carbamoyltryptamine (5-CT, 1),[16] 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)indole (RU 24969, 2),[17] 1-(3-acetamidophenyl)piperazine (BEA 1654, 3)[18] and several putative antagonists such as propranolol, pindolol and cyanopindolol.[19-24] In general, the above-mentioned drugs have similar affinities for both the 5-HT_{1A} and 5-HT_{1B} subtypes of the 5-HT_1 recognition site, and cannot be used as tools to distinguish between them.[16]

5-HT_{1A} Recognition sites : identification and functional correlates - The functional effects of stimulation of the putative 5-HT_{1A} receptor have



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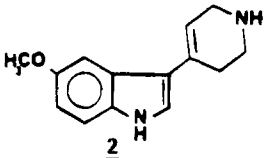

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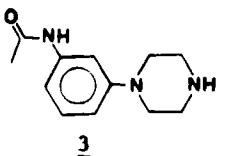

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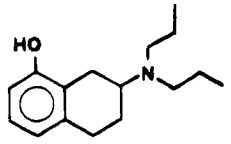
received considerable attention over the last 3 years. Of crucial importance to these studies was the finding that the tetralin derivative, 8-OH-DPAT (4), is a selective agonist at the putative 5-HT_{1A} receptor.[25] In radioligand binding studies this compound proved to be >600 times more potent at 5-HT_{1A} sites than at 5-HT_{1B} or 5-HT_2 sites.[26,27] Tritiated 4 has been prepared and is now used to label selectively the 5-HT_{1A} recognition site.[28] Compound 4 has many central and peripheral actions including effects on the cardiovascular system,[29] feeding behavior,[30] sexual activity,[31] the startle response[32] and body temperature.[33] Attempts have been made to confirm the receptor type involved in the functional responses to 8-OH-DPAT. For example, the potent stimulant effect on adenylate cyclase activity in guinea pig hippocampus homogenates is blocked by spiperone, a potent 5-HT_2 receptor antagonist which has 5-HT_{1A} receptor antagonist activity, but not by the more selective 5-HT_2 receptor antagonist, ketanserin, nor by the 5-HT_3 receptor antagonist, 1αH, 3α, 5αH-tropan-3-yl-3,5-dichlorobenzoate (MDL 72222).[34] Electrophysiological studies in the rat dorsal hippocampal slice preparation indicate that the decreases in the CA1 population spike amplitude, induced by 5-HT or 8-OH-DPAT, are also mediated by the 5-HT_{1A} receptor.[35] Contraction of the canine basilar artery induced by 8-OH-DPAT appears similarly to be mediated by 5-HT_{1A} receptors.[36] These *in vitro* studies are complemented by *in vivo* experiments which demonstrate that certain of the behavioral effects of 8-OH-DPAT are the result of the stimulation of 5-HT_{1A} receptors, as are its effects on body temperature.[37-39] The inhibition of transmitter release following field stimulation of the guinea pig ileum is a functional correlate of the 5-HT_{1A} recognition site in the periphery.[40]



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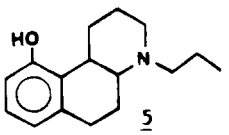
5-HT_{1A} Recognition sites : new ligands - Compounds with appreciable affinity for the 5-HT_{1A} recognition site can be classified under the following headings:

Tetralin derivatives - Compound 4 was of particular interest in that its tetralin structure was chemically distinct from earlier classes of 5-HT receptor agonists. The position of the hydroxy substituent is crucial for the potency and selectivity of the compound at the 5-HT_{1A} site. Thus, whereas 4 has no effect on central catecholamine receptors, 5-, 6- or 7-hydroxylated derivatives have powerful dopamine receptor agonist activity.[41] Many other tetralin analogues have been prepared and tested for activity at central 5-HT receptors using biochemical and behavioral tests in rats.[42] Quantification of this activity by measuring changes in 5-HTP turnover in limbic structures, striatum and cortex allowed the following conclusions: (i) O-methylation of 4 results in a 6-fold decrease in activity; (ii) modification of the amino substituent gives the order of potency -NH-nBu < -NH-iPr << -NH-Me ~ -piperidine < $-NMe_2$ <-NH-Et < -NH-nPr < $-NEt_2$ < $-N(nPr)_2$; (iii) the R(+) enantiomer of 4 is slightly, but significantly, more potent than the S(-) enantiomer. This last observation has been confirmed by direct measurement of the affinity for the 5-HT_{1A} recognition site in rat brain cortex.[43] Compounds 5 to 7 have also been prepared and shown to have agonist activity at central 5-HT receptors.[44,45]



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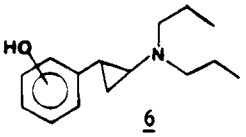

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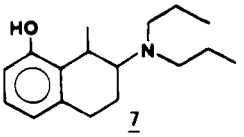

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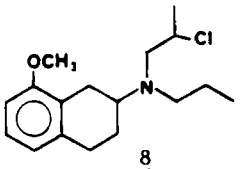
Two putative 5-HT_{1A} receptor alkylating agents 8 and 9, have been synthesised.[46,47] Of these, 8 is reported to bind selectively and irreversibly to the [^{3}H]-8-OH-DPAT recognition site, but these findings remain to be confirmed by functional studies.[46] The other derivative, 9, is a selective antagonist of the cardiovascular response to 8-OH-DPAT but the effects are not irreversible.[47]



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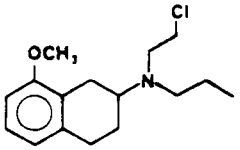

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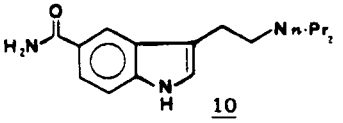

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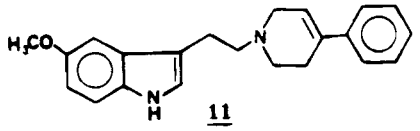
Indole derivatives - As mentioned above, RU 24969 (2) has high affinity for the 5-HT_1 recognition site,[48] but it shows only limited capacity to discriminate between the 5-HT_{1A} and 5-HT_{1B} subtypes.[43,49] Nevertheless, its structure is of interest since the tetrahydropyridine ring prevents superposition of the basic nitrogen and the indole ring with the same structural features in 5-HT or lysergic acid diethylamide (LSD). Various analogues have been examined for their ability to decrease rat brain-stem 5-hydroxyindoleacetic acid concentrations.[50] Several analogues of 5-methoxytryptamine (N,N-dimethyl, N,N-diethyl and N,N-di-n-propyl-5-methoxytryptamine) have good affinities and some selectivity for the 5-HT_{1A} recognition site.[51] There is a correlation between their capacity to discriminate between the 5-HT_{1A} and 5-HT_{1B} sites and the length of the N-alkyl substituent. This trend was also evident with analogues of 5-CT ; for example the N-di-n-propyl derivative 10 (DP-5-CT) had similar affinity to 5-CT for the 5-HT_{1A} binding site but was much more selective.[43] DP-5-CT is the most potent and selective 5-HT_{1A} site ligand described to date. Two other indole derivatives, 11 and 12, have recently been disclosed as potent and relatively selective 5-HT_{1A} ligands, suggesting that more extended N-substitution does not necessarily lead to a loss of affinity.[52]



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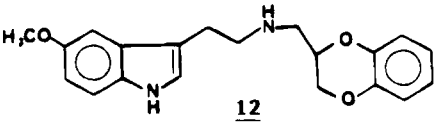

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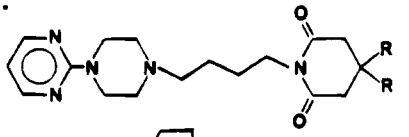
Aryl piperazine derivatives - The activity of BEA 1654 (3) has already been mentioned.[43] Buspirone (13), a clinically effective anxiolytic drug, is an example of an aryl piperazine derivative with affinity and selectivity for the 5-HT_{1A} sites.[53] It has moderate effects on dopamine and α_1-adrenoceptors, but negligible activity on α_2, β, muscarinic, 5-HT_2, γ-aminobutyric acid or benzodiazepine receptors.[54] It has been suggested that the anxiolytic properties of buspirone arise through an interaction at 5-HT_{1A} sites.[55-57] Gepirone (14), an analogue of buspirone which is devoid of significant effects at dopamine receptors,[58] has recently been shown to have selective activity at the 5-HT_{1A} recognition site.[59] Many other structural analogues of buspirone have been described, but no structure activity study involving affinities for the 5HT_{1A} site has yet been reported.[60] TVX Q 7821 (15), a compound which contains the saccharin moiety, binds with reasonable affinity to

the 5-HT_{1A} recognition site of rat frontal cortex.[61] [^{3}H]-TVX Q 7821 has been prepared and appears to be a useful ligand for the 5-HT_{1A} recognition site.[62]



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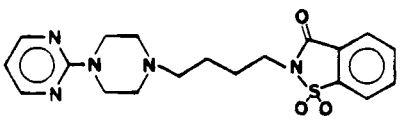

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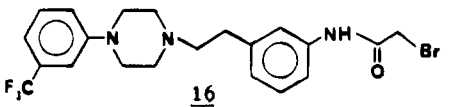

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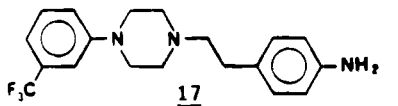

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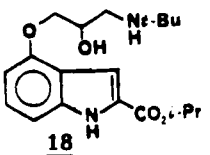
1-[3-(Trifluoromethyl)phenyl]piperazine has greater affinity for the 5-HT_{1B} site than for the 5-HT_{1A} site.[63] In contrast, two of its derivatives, 16 and 17, are reported to display both high affinity and selectivity for the 5-HT_{1A} site, providing a further example of a shift in the selectivity pattern of a 5-HT receptor substrate towards 5-HT_{1A} activity as a result of N-substitution.[63-65] Finally, the α_1-adrenoceptor ligand [^{3}H]-WB 4101 labels the 5-HT_1 receptor with high affinity : its efficient displacement by spiperone[66] suggests that the 5-HT_{1A} subtype is involved.

5-HT_{1B} recognition sites : identification and functional correlates - A limited number of compounds has been shown to have a degree of selectivity for the 5-HT_{1B} recognition site. These are RU 24969 (2),[48,67] the indolyloxypropanolamines 18[16,21] and 19,[21] and several piperazine derivatives.[67] A number of other compounds including tryptamines,[16] some β-adrenoreceptor antagonists,[16,20] and methiothepin[16] displays affinity but no selectivity for the 5-HT_{1B} recognition site. The 5-HT_{1B} recognition site can be labeled selectively with 3-[^{125}I]-2-cyanopindolol (19)[21,68] in the presence of 30 μM isoprenaline. The autoreceptor of the terminal 5-HT neurones appears to be the functional correlate of the 5-HT_{1B} recognition site. Thus, compounds which show affinity for the 5-HT_{1B} site have effects at the 5-HT autoreceptor. For example 2-cyanopindolol (20) is a potent 5-HT autoreceptor antagonist, although it does not discriminate between the 5-HT_{1A} and 5-HT_{1B} subtypes.[16,23,24] RU 24969, which has good affinity for the 5-HT_{1B} site, is a potent autoreceptor agonist whereas 8-OH-DPAT which is selective for the 5-HT_{1A} site is inactive.[48,69] Correlation analysis of a range of some 30 drugs of diverse structures, with affinity and various degrees of selectivity at the central 5-HT recognition sites, has established that the terminal 5-HT autoreceptor does indeed belong to the 5-HT_{1B} subtype.[16] There appears to be a functional relationship between the 5-HT autoreceptor and the presynaptic 5-HT reuptake site. Thus, it has been clearly demonstrated that a number of 5-HT uptake blockers can reduce the efficacy and potency of 5-HT autoreceptor agonists.[70,71] The 5-HT autoreceptor located on the 5-HT neuronal cell bodies in the dorsal raphe[72] is an interesting exception and may be of the 5-HT_{1A} type.



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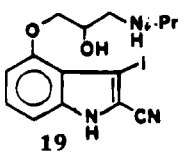

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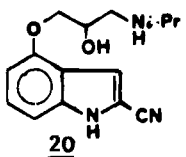

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5-HT_{1C} Recognition sites : identification and functional role - Palacios and his colleagues were the first to define a subclass of the 5-HT recognition site which could not be classified as either 5-HT_{1A}, 5-HT_{1B} or 5-HT_2.[13,73] These sites, which are concentrated particularly in the choroid plexus, have been designated 5-HT_{1C}.[13,14] The 5-HT_{1C} recognition sites can be labeled by [^{3}H]-mesulergine with the use of 5-HT or mianserin to define non-specific binding.[68] Both receptor binding and autoradiographic studies have established that the choroid plexuses of rat, pig and man are especially rich in 5-HT_{1C} sites and that significant numbers exist in other brain areas including frontal cortex and hippocampus.[13,14] Several established 5-HT receptor antagonists show nanomolar affinity for 5-HT_{1C} sites including metergoline, mianserin, LSD, cyproheptadine, methiothepin and mesulergine ; none is, however, selective.[13,16] Agonists have generally lower affinities and several, including 4-hydroxytryptamine, 5-aminotryptamine and tryptamine, show a degree of selectivity for the 5-HT_{1C} site.[16] A functional role for the 5-HT_{1C} recognition site remains to be defined. However, since they are mainly located in the choroid plexus, they may be involved in the control of the volume and composition of the cerebrospinal fluid.[74]

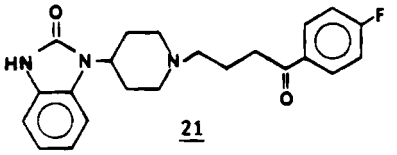
5-HT_2 Recognition sites : identification and functional correlates - The 5-HT_2 recognition site was first characterized in radioligand binding studies using the tritiated neuroleptic [^{3}H]-spiperone.[10] However this ligand is less than ideal since its affinity for the dopamine D_2 receptor is 33 times higher than that for the 5-HT_2 receptor (Table).[8,75] Several other radioligands have since been evaluated in an attempt to achieve both high affinity and selectivity for the 5-HT_2 site. Most have proven unsatisfactory.[10,76-81] Although [^{3}H]-ketanserin[82] is only 25 times less active at H_1-histamine and α_1-adrenoceptors than 5-HT_2 receptors, it is the radioligand most commonly used to label the 5-HT_2 sites, with mianserin present to define the non specific binding.[16] A recent paper identifies N_1-methyl-2-[^{125}I]-LSD (31) as having the highest affinity yet reported and the best overall selectivity for the 5-HT_2 receptor.[83]

Administration of 5-HT precursors or directly acting agonists results in head twitch in the mouse. The original postulate that this response is mediated by 5-HT_2 receptors[84] has received general[85-87] but not universal support.[4] The 5-HT_2 receptor antagonists are able to block the discriminative stimulus properties of hallucinogenic compounds, such as LSD and 1-[2,5-dimethoxy-4-methylphenyl]-2-aminopropane, leading to the suggestion that the mechanism of action of these agents involves 5-HT_2 receptor related events.[88] Electrophysiological studies of the facial and spinal motoneurones indicate that the facilitatory effect of 5-HT or 5-HT receptor agonists on the depolarizing action of excitatory amino acids is mediated by a 5-HT_2 receptor.[89,90] Similarly, from antagonist studies, the 5-HT_2 receptor appears to mediate the depolarisation of cell bodies of sympathetic preganglionic neurones evoked by 5-HT.[91]

The clinical significance of drugs with selective actions at 5-HT_2 receptors remains unclear. The initial indication that 5-HT_2 receptor antagonists would be hypotensive has now been largely discounted.[92,93] In animal studies, chronic administration of antidepressant drugs or electroconvulsant treatment leads to a decrease in 5-HT_2 receptor number and a role for 5-HT_2 receptor antagonists in affective disorders has been postulated.[94] Early clinical studies with the 5-HT_2 antagonist, ritanserin, suggest an anxiolytic activity in this class of drug.[95]

5-HT_2 Recognition sites : new ligands - A number of new 5-HT_2 receptor ligands has been characterized in binding assays and/or relevant functional tests. The table includes the details of the activities and selectivities of these compounds in comparison with those of established 5-HT_2 receptor antagonists. They can be classified as follows :

Butyrophenone derivatives - Many butyrophenone derivatives were described in the earlier review as having high affinity for the 5-HT_2 recognition site.[1] Additions to this list include benperidol (21), a close analogue of spiperone which is only slightly less potent in the [^{3}H]-ketanserin binding assay.[82]



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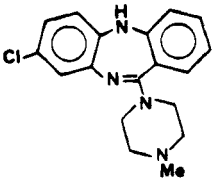
Tricyclic compounds - Compounds classified under this heading include cyproheptadine, pizotifen and mianserin, all of which show high affinity for the 5-HT_2 binding site. The neuroleptic clozapine (22) has been shown to be an antagonist of 5-HT_2 receptors in vitro and in vivo.[82,96] Clopipazan (25) has very high affinity for [^{3}H]-ketanserin labeled sites.[82] WAL 1307 (24) is active in functional tests of 5-HT_2 receptor activity.[97]



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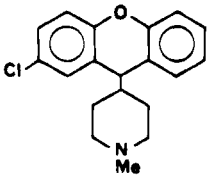

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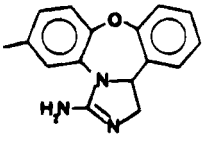

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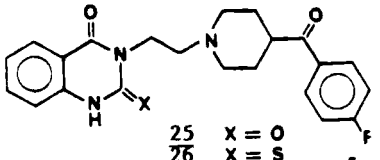
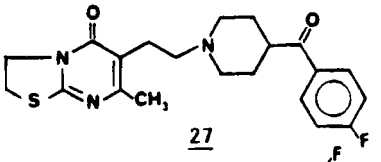
Ketanserin derivatives - Several new analogues of ketanserin (25) have recently been described : altanserin (26), setoperone (27), R 56413 (28) and ritanserin (29). All display very high affinity for the 5-HT_2 receptor with different profiles vis-à-vis other receptors. All are undergoing clinical trial in CNS disorders.[98] Ritanserin is of particular interest as an extremely potent, long acting and centrally-active 5-HT_2 receptor antagonist which shows anxiolytic effects in preliminary clinical trials.[95,98] 7-Azido-ketanserin has been reported to bind irreversibly to the 5-HT_2 recognition sites, although it also shows marked affinity for H_1-histamine receptors.[99,100]



25 X = O
26 X = S



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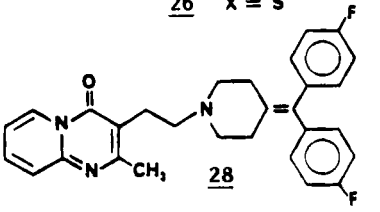

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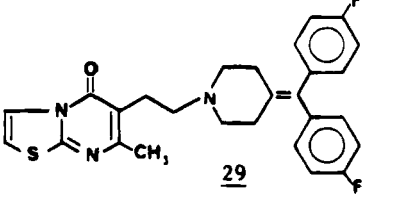

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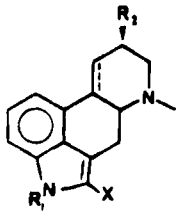
Table - 5-HT_2 receptor affinities determined in [^{3}H]-ketanserin binding studies. (K_i values for the 5-HT_2 receptor are in nM ; selectivities are expressed as ratios of K_i values).

Compounds	5-HT_2	$5\text{-HT}_1/5\text{-HT}_2$	$H_1/5\text{-HT}_2$	$\alpha_1/5\text{-HT}_2$	$\alpha_2/5\text{-HT}_2$	$D_2/5\text{-HT}_2$
Ketanserin Derivatives						
Altanserin[98]	0.16	> 6000[a]	200	25	620	350
Ritanserin[98]	0.19	> 5000[a]	125	185	315	115
R 56413[98]	0.26	> 3000[a]	75	280	250	480
Pirenperone[82]	0.28	-	-	-	-	-
Setoperone[98]	0.37	> 2500[a]	145	35	65	70
Ketanserin[98]	0.39	> 2500[a]	25	25	> 2500	565
Butyrophenone Derivatives						
Spiperone[8,82]	0.53	190	> 1800	18	> 1800	0.3
Pipamperone[82]	0.78	-	-	-	-	-
Benperidol[82]	1.20	-	-	-	-	-
Ergoline Derivatives						
2-iodo-LSD[80]	0.9	35[a]	1300	110	110	44
Metergoline[8,82]	0.28	46	3900	135	1300	80
Methysergide[82]	0.94	-	-	-	-	-
d-LSD[8,82]	2.50	8	> 440	65	35	8
Mesulergine[73]	3.16	-	-	-	-	-
Me-I-LSD[83]	0.26[b]	180	-	60	280	34
LY 53587[101]	0.05[b]	No effect	260000	5800	-	-
Tricyclic Derivatives						
Pizotifen[82]	0.28	-	-	-	-	-
Clopipazan[82]	0.39	-	-	-	-	-
Methiothepine[8,82]	0.39	95	14	1	80	10
Cyproheptadine[82]	0.44	-	-	-	-	-
Mianserin[8,82]	1.4	710	2.0	58	43	442
Miscellaneous						
Clozapine[82]	2.6	-	-	-	-	-
Xylamidine[82]	1.5	-	-	-	-	-
Cinanserin[82]	2.0	-	-	-	-	-

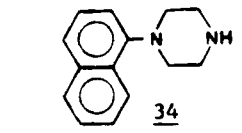
a: Selectivity versus the high affinity 5-HT_1 site.[19]
b: Dissociation constant

Ergoline derivatives - The classical 5-HT receptor antagonists, LSD, methysergide and metergoline have been joined recently by several new highly active compounds containing the ergoline skeleton : 2-iodo-LSD (30),[80] N$_1$-methyl-2-iodo-LSD (31),[83] mesulergine (32),[73,102] and LY 53857 (33).[101] All these compounds show a degree of selectivity for the 5-HT_2 site. LY 53857, which lacks α_1-adrenoceptor and dopamine receptor antagonist activities, shows activity in animal models predictive of antidepressant activity.[101,103]



	X	R_1	R_2	△
30	I	H	$CONEt_2$	
31	I	Me	$CONEt_2$	
32	H	Me	$NHSO_2NMe_2$	HH
33	H	i-Pr	$CO_2CH(Me)CHOH\text{-}CH_3$	HH

Miscellaneous - Diverse chemical structures have been reported to have 5-HT_2 receptor antagonist properties. For example, 1-(1-naphthyl)-piperazine (34) shows good affinity for the 5-HT_2 receptor and, being some 2000 times less active at α_1-adrenoceptors, is markedly selective.[104] Two other aryl piperazine derivatives, TR 2515 (35)[105] and trazodone (36),[106] are also 5-HT_2 receptor antagonists. The benzazepine 37 (SCH 23390), which is a dopamine D_1-receptor antagonist, inhibits [^{3}H]-spiperone binding in rat cortex with an IC_{50} of 112 nM.[107] Verapamil, a class II calcium-antagonist, had an IC_{50} of 290 nM in the same test.[108-110] Two related amidines, xylamidine (38) and BW 501C67 (39), display high affinity for the 5-HT_2 receptor.[82] BW 501C67 discriminates effectively between 5-HT_2 and α_1-adrenoceptors and is remarkable in being unable to block central 5-HT_2 receptors.[111,112]



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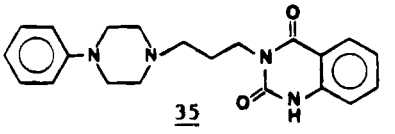

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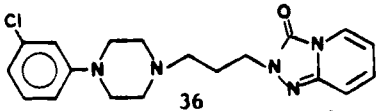

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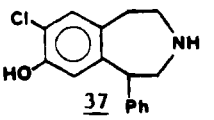

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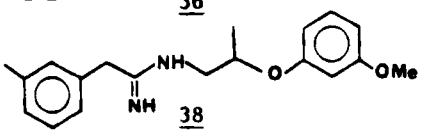

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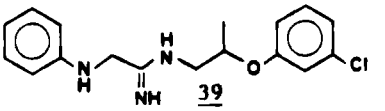

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Chapter 6. Formation and Degradation of Neuropeptides

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Introduction* - Synaptic communication was long thought to be mediated solely by classical neurotransmitters such as monoamines, amino acids and acetylcholine. In the last decade, however, an increasing number of peptides has been found in CNS neurons.[1-3] Neuropeptides can be defined as those peptides which affect communication between neurons either by direct transmitter action or indirectly via neuromodulation.[4] The availability of newer diagnostic and analytical techniques has greatly facilitated the identification of such peptides, many of which had already been described in the gastrointestinal tract, pancreas and pituitary. A growing number of neuropeptides has been identified through gene sequencing of their peptide precursors. The neuroanatomical localization of many of the neuropeptides listed in Table 1 is described by Nieuwenhuys.[1]

The situation is further complicated by the frequent coexistence of neuropeptides with each other (eg. CCK and VIP, CCK and NT, VIP and NT, CRF and AVP, CRF and OT, TRH and SP, OT or AVP and enkephalins) or with classical neurotransmitters (eg. CCK and dopamine, AVP and norepinephrine, NPY with epinephrine or norepinephrine, SP or TRH with 5-HT, SS with norepinephrine and GABA, and NT or VIP with acetylcholine).[1,5,6] Cotransmitters may greatly increase the number of chemical signals available for neuronal communication. Neuropeptides are involved in homeostatic systems, including the regulation of pain, blood pressure, temperature, thirst, feeding, learning, memory and trophic function.[2] Neuropeptides have also been implicated in neurological diseases[7] such as Alzheimer's disease,[8] schizophrenia,[9] Huntington's chorea,[9] and epilepsy,[10] as well as in the immune system.[11] The amino acid sequences of the majority of characterized neuropeptides are given in Table 2.

Biosynthesis and post-translational processing - Naturally occurring peptides are biosynthesized either directly from amino acids, by more or less peptide specific enzymes, or by cleavage (processing) of precursor proteins synthesized at the level of the ribosome. Peptides of the

*Peptide abbreviations used in this chapter are: ACTH, adrenocorticotropic hormone; CCK, cholecystokinin; CGRP, calcitonin-gene-related peptide; CLIP, corticotropin-like intermediate lobe peptide; CRF, corticotropin releasing factor; DSIP, delta-sleep-inducing peptide; FMRF and GAWK are names, not abbreviations; GHRH, growth hormone-releasing hormone; GRP, gastrin-releasing peptide; LPH, lipotropin; LHRH, luteinising hormone-releasing hormone; MSH, melanocyte stimulating hormone; NPY, neuropeptide Y; NT, neurotensin; ODN, octadecaneuropeptide, fragment of diazepam binding inhibitor; OT, oxytocin; POMC, pro-opiomelanocortin; SP, Substance P; SS, somatostatin; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; VP, vasopressin.

Table 1. Neuropeptides in Mammalian CNS

Neuropeptides[a]	Chain Length of Most Abundant Form[b]	Neuropeptides[a]	Chain Length of Most Abundant Form[b]
ACTH	39	Kyotorphin	2
Amidorphin[12]	26	ß-LPH	89
Angiotensin	8	Locust Adipokinetic hormone[19]	10
Atrin[13,c]	28	LHRH (GnRH)	10
Bombesin[14,d]	14	α-MSH (see also Ref.20)	13
Bradykinin	9	γ_3-MSH[21]	26
Calcitonin	32	Motilin	22
αCGRP	37	α-Neoendorphin	10
ßCGRP[15]	37	ß-Neoendorphin	9
Carnosine (ßAla-His)	2	Neokyotorphin[22]	5
CCK	8	Neuromedin B-32[23]	32
CRF	41	Neuromedin U-25; U-8[24]	25;8
Cyclo(His-Pro)[16]	2	Neuropeptide K[25,26;e]	36
DSIP	9	NPY (see also Ref.27)	36
Dynorphin A	17	NT	13
Dynorphin B-29 (leumorphin)	29	ODN[28]	18
Dynorphin B (rimorphin)	13	OT	9
ß-Endorphin	31	Prosomatostatin fragments[29]	76,44
Leu-Enkephalin	5	Secretin	27
Met-Enkephalin	5	SS	14
FMRF-amide	4	SP	11
Galanin[17]	29	Synenkephalin[30]	70
Gastrin	17	TRH	3
GAWK[18]	74	VIP	28
Glucagon	29	VP	9
GHRH (somatocrinin)	44		
Hydra head activator	11		
Insulin	51		

(a) putative neuropeptides are referenced; (b) human where available, but other biologically active fragments exist; (c) see chapter titled "Atrial Natriuretic Factor" by R. W. Lappe and R. L. Wendt which is included in this volume (Ch. 27); (d) probably GRP; (e) C-terminal decapeptide also called neurokinin A or α, neuromedin L or substance K.

first group are usually small and often contain amino acids which are not found in proteins; carnosine (Table 1) and glutathione are examples. In the case of the second group an initial translational product, the "pre-protein", contains a signal sequence at its N-terminus which can be removed by a "signal peptidase" to yield the "pro-protein". This in turn serves as the precursor molecule for component peptides. The concept of this post-translational proteolytic cleavage of large precursors was based on pulse-chase studies with radioactive amino acids.[2,31-33] Application of recombinant DNA techniques has subsequently elucidated the amino acid sequences of many neuropeptide precursor proteins.[34] However, precursor proteins may contain several biologically active products. For instance, pro-opiomelanocortin (POMC) was shown to be the precursor protein for ACTH, ß-LPH and γ_3-MSH; smaller neuropeptides were found upon further processing of ACTH and ß-LPH, each with their own characteristic pattern of biological activity (see Fig. 1).[35] Pro-enkephalin A, on the other hand, contains four copies of Met-enkephalin and one copy each of Leu-enkephalin, Met-

enkephalin-Arg-Phe-OH and Met-enkephalin-Arg-Gly-Leu-OH; in addition to these molecules several larger enkephalin-containing peptides have been found, all with the opioid-like activity.[36]

In the first well-documented case, insulin, it was found that cleavage took place at the carboxyl site of paired basic amino acid residues in the pro-protein, followed by enzymatic removal of these basic amino acids. Cleavage at paired Lys and/or Arg residues was later found to be characteristic for many precursors. Precursors of the following neuropeptides are processed in this way: ACTH (Fig. 1); calcitonin and CGRP;[37] CCK (at the C-terminal end of CCK-8);[38] CRF;[39] dynorphin A and the other peptides generated from prodynorphin (= proenkephalin B), such as Leu-enkephalin, α- and ß-neoendorphin and dynorphin A(1-8);[40] ß-endorphin (Fig. 1); Met- and Leu-enkephalin;[36] gastrin;[41] glucagon[42] (two glucagon-like peptides are also present between doublets of basic amino acid residues in human preproglucagon); insulin;[43] ß-LPH (Fig. 1); α-MSH (Fig. 1); SS-14;[44] SP (bovine, at C-terminal end);[45] substance K (the C-terminal decapeptide of bovine neuropeptide K);[45] TRH (rat)[46] and VIP[47] (a closely related peptide of 27 amino acids, PHM, is also present). Cleavages at a single arginine are also known; for example in the precursors of dynorphin B (rimorphin),[40] CCK-8,[38] SS to give the 28-peptide[44] and bradykinin.[48] No precursor (sequence) has been found for FMRF-amide, GHRH, hydra head activator, kyotorphin, LHRH, motilin, NT, NPY, DSIP, proctolin (insects) and secretin, although in several cases immunoreactive, high molecular mass, material has been identified (eg. for motilin and DSIP). In other cases the presence of an N-terminal pyroglutamic acid residue (eg. NT, LHRH) and/or a C-terminal amide (FMRF-amide, GHRH, LHRH and NPY) strongly suggests post-translational modification of a precursor (see below).

Modifications to pro-proteins which occur before cleavage include disulfide bridge formation, glycosylation, sulfation, methylation and possibly phosphorylation.[32,49,50] CCK (large and small forms) contains a sulfated tyrosine in the C-terminal region which is essential for secretion of pancreatic enzymes and gall-bladder emptying but not for certain CNS activities. About half of the gastrin-related peptides are sulfated but this has no effect on gastric acid secretion in mammals. A small amount of Leu-enkephalin from rat striatum is sulfated but it is biologically inactive. Very little is known about the sulfotransferase activities involved in the tyrosine sulfation step. Phosphorylation of ACTH and CLIP has been found at serine 31, where the sequence -Ser-X-acidic- appears to be part of a recognition site.[32] Biosynthetic studies indicate that phosphorylation and glycosylation occur at about the same time, shortly after the synthesis of POMC; neither process seems to influence the biological activity greatly.[32]

Post-translational modification reactions which occur after cleavage of the peptide from its precursor include C-terminal amidation, N-terminal acetylation and pyroglutamic acid formation.[31-33,49,50] In the first-mentioned case, cleavage at doublets of basic amino acid residues by trypsin-like enzymes[31,33,51] is followed by removal of these basic amino acids by carboxypeptidase B-like proteases.[31,33,52,53] In all known cases there is a glycine residue at the N-terminal end of the basic doublet, which is thought to serve as a recognition site for amidation. Human plasma, CSF and CNS tissue contain enzymes with "peptide α-amidation" activity.[33,49,54,55] Examples of -X-Gly-Lys(or Arg)-Lys(or Arg)- systems that result in

-X-NH_2 are calcitonin, CCK, gastrin, α-MSH, OT, SP, TRH, VIP and VP. Acetylation of the N-terminal end, also enzymatic,[32,33] can influence the biological activity; for example, a much more potent stimulation of melanocytes is seen with N^{α}-acetylated ACTH-(1-13)-NH_2, (ie. α-MSH), whereas a drastic loss of affinity for opiate receptors has been observed with acetylated ß-endorphin. Formation of pyroglutamic acid residues from N-terminal glutamine residues is another modification.[56] This process, well known in peptide synthesis, was originally thought to take place spontaneously but enzymatic cyclization has been reported: examples are bombesin, LHRH, NT and TRH. It has also been claimed that glutamic acid can be cyclized in vivo to pyroglutamic acid.[56] These N- and C-terminal modifications may influence the biological activity and confer resistance to enzymatic degradation.

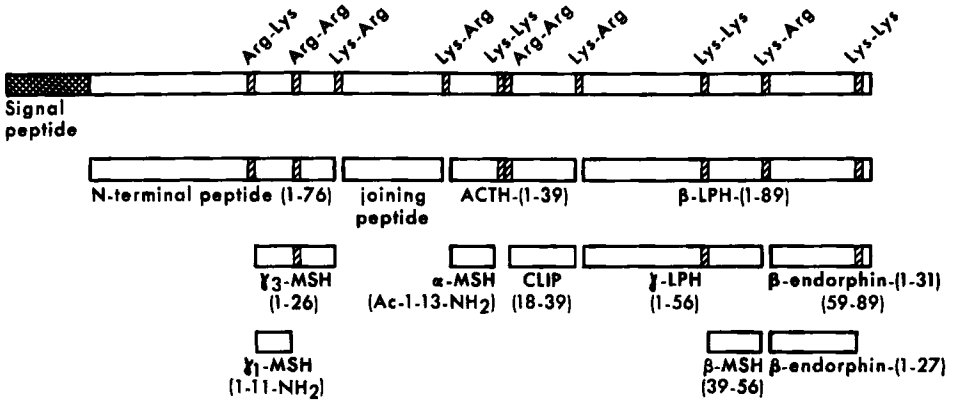

Fig. 1. Schematic representation of the human POMC precursor molecule and products of its cleavage at paired basic amino acid residues. The precursor is comprised of 267 amino acids.[57] A POMC-converting enzyme, specific for the Lys-Arg sequences in POMC, has been isolated from bovine pituitary intermediate lobe secretory vesicles.[58] One or two sites in the N-terminal part of the precursor are glycosylated. [Other endogenous peptides contained in the precursor are the ß-LPH fragment 1-75 (and perhaps 1-74); the ß-endorphin fragments 1-26, 1-21, 1-17 (γ-endorphin), 1-16 (α-endorphin) and their N^{α}-acetylated forms; and N^{α}-acetylated ß-endorphin (1-31) and (1-27); ß-endorphin-(1-18) has been isolated from human pituitaries.[35,59,60] The absence of pairs of basic amino acids at appropriate positions in the precursors implies a different processing to that indicated in Fig. 1.]

It has been postulated that there is a limited number of converting enzymes with a wide cellular distribution and that the secondary structure of the substrate may direct the specificity of the processing.[61] This might explain why some pairs of basic residues in a molecule are processed while others are not, eg. in CRF, glucagon, NT and VIP. Moreover, conformational factors coupled with specific enzyme requirements, may explain why the ratios of ACTH:α-MSH and ß-LPH:ß-endorphin are so different for processing of POMC in different brain regions.[62-64] Further evidence for this hypothesis is found in the formation of ß-endorphin from ß-LPH.[65] However, the tissue-specific processing of precursor molecules can be influenced not only by structural features but also by physiological (eg. stress[66]) and pharmacological stimuli.[59,67-70] Thus, chronic administration of haloperidol to rats

leads to a marked increase in the levels of ß-endorphin-related peptides in the pars intermedia and brain stem but has no effect in the hypothalamus and anterior pituitary.[59] The increase is, however, confined to the non-opioid N^{α}-acetylated ß-endorphin 1-27 and 1-26 fragments. Increases in biosynthesis and processing of proenkephalin A have also been reported following (sub)chronic, but not acute, treatment with haloperidol.[67] Chronic administration of antidepressants[68] or oral contraceptives[69] enhances rat brain opioid activity, and antidepressants additionally raise CSF and regional brain levels of TRH.[70]

Enzymatic degradation - Since there are so many different neuropeptides in the brain it is unlikely that separate mechanisms exist for the inactivation of each peptide at its particular synapse.[71,72] Unlike classical neurotransmitters whose synaptic action is largely terminated by reuptake mechanisms, neuropeptides in the CNS generally undergo extra-cellular degradation by membrane-bound enzymes present near pre- or post-synaptic peptide receptors.[2,72] These enzymes may have the potential to regulate the amount of peptide released or available at the site of action, to control the duration of action, and to guide the biotransformation.[73,74] This last-mentioned function of the enzymes can involve formation of a biologically active peptide from the parent neuropeptide or conversion of an inactive precursor to an active form. The functional significance of biotransformation is evident from the number of neuropeptides whose enzymatically formed metabolites also have effects (similar, opposite or different) in the body[35,72-78] Examples include ACTH, opioid peptides, CCK, LHRH, VP, OT and SP. As with neuropeptides, enzymes may play a role in disease states. Two recent examples are the exopeptidase angiotensin converting enzyme (ACE), which was found to be significantly higher in the serum of patients with untreated Addison's disease,[79] and proline endopeptidase[80] which was abnormally low in certain areas of the brain of patients with Huntington's chorea.[81]

Both exo- and endopeptidases are involved in the degradation of neuropeptides.[82-84] Since several neuropeptides have blocked end groups (N^{α}-Ac; pGlu; C-terminal amide), endopeptidases are commonly required to initiate hydrolysis. Endopeptidases are classified into serine-, thiol-, carboxyl- and metallo-endopeptidases. No satisfactory classification on the basis of their specificity is possible and so the identity of the essential catalytic group of the enzyme is used for most classes. Endopeptidases with unknown mechanisms are grouped in an additional class. The categories, and their subdivisions, have all been given numbers.[83,84] On the basis of their substrate specificities the major classes of exopeptidases are: aminopeptidases which cleave at the N-terminal [H-X↓Y-] (subdivided into several classes depending on the nature of X and Y, eg. pyroglutamate peptidase is selective for pGlu containing peptides); carboxypeptidases which cleave at the C-terminal [-Y↓Z-OH] (eg. lysosomal carboxypeptidases whose specificity is based on the nature of Z); dipeptidases which cleave dipeptides into amino acids; dipeptidylpeptidases which cleave at the N-terminal [H-X-Y↓Z-] (subdivided into classes I-IV depending upon the nature of X and Y); and peptidyldipeptidases which cleave at [-X↓Y-Z-OH] (eg. ACE which removes the C-terminal dipeptide from angiotensin I).

Although there are now detailed studies on the localization of many neuropeptides in the CNS, much less is known about the distribution of peptidases.[1-3] Enzymes which have been found in brain membranes (and in many other mammalian tissues) include: endopeptidase 24.11 which has

Table 2. The Amino Acid Sequences of Neuropeptides.

(Peptides are of human origin unless stated otherwise. Carnosine and cyclo[His-Pro] are already included in Table 1. The primary structure of synenkephalin is not yet known. For longer peptides the one-letter code is used.[85])

ACTH : H-Ser^{1}-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe^{39}-OH

Amidorphin (bovine) : H-Tyr^{1}-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu^{26}-NH_2

Angiotensin I : H-Asp^{1}-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu^{10}-OH; angiotensin II is 1-8.

Atrin : H-Ser^{1}-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr^{28}-OH

Bombesin (amphibian) : pGlu^{1}-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met^{14}-NH_2

Bradykinin : H-Arg^{1}-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg^{9}-OH

Calcitonin : H-Cys^{1}-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro^{32}-NH_2

αCGRP (rat) : H-Ser^{1}-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe^{37}-NH_2; in βCGRP there is Lys^{35}

CCK : H-Val^{1}-Ser-Gln-Arg-Thr-Asp-Gly-Glu-Ser-Arg-Ala-His-Leu-Gly-Ala-Leu-Leu-Ala-Arg-Tyr^{20}-Ile-Gln-Gln-Ala-Arg-Lys^{26}-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp^{51}-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe^{58}-NH_2; CCK-8 is 51-58; CCK-33 is 26-58; CCK-39 is 20-58.

CRF : H-Ser^{1}-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile^{41}-HN_2

DSIP (rabbit) : H-Trp^{1}-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu^{9}-OH

Dynorphin A : H-Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile^{8}-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln^{17}-OH; dynorphin A(1-8) is the N-terminal octapeptide.

Dynorphin B-29 : H-Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr^{13}-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Ser-Gly-Glu-Leu-Phe-Asp-Ala^{29}-OH; dynorphin B is 1-13.

ß-Endorphin : H-Tyr^{1}-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu^{31}-OH (see Fig. 1).

Leu-Enkephalin : H-Tyr^{1}-Gly-Gly-Phe-Leu^{5}-OH

Met-Enkephalin : H-Tyr^{1}-Gly-Gly-Phe-Met^{5}-OH

FMRF-amide (mollusc) : H-Phe^{1}-Met-Arg-Phe^{4}-NH_2

Galanin (pig) : H-Gly^{1}-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys-Tyr-Gly-Leu-Ala^{29}-NH_2

Gastrin (pig) : pGlu^{1}-Leu-Gly-Leu-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Leu-Ala-Lys-Lys-Gln^{18}-Gly-Pro-Trp-Met-(Glu)$_5$-Ala-Tyr(SO_3H)-Gly-Trp-Met-Asp-Phe^{34}-NH_2; gastrin 17 is [pGlu^{18}]18-34.

GAWK : F^{1}-L-G-E-G-H-H-R-V-Q-E-N-Q-M-D-K-A-R-R-H-P-Q-G-A-W-K-E-L-D-R-N-Y-L-N-Y-G-E-E-G-A-P-G-K-W-Q-Q-Q-G-D-L-Q-D-T-K-E-N-R-E-E-A-R-F-Q-D-K-Q-Y-S-S-H-H-T-A-E^{74}

Glucagon : H-His^{1}-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr^{29}-OH

Table 2 (contd.)

GHRH : H-Tyr^{1}-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu^{44}-NH_2

Hydra head activator : pGlu^{1}-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe^{11}-OH

Insulin : A-chain G^{1}-I-V-E-Q-C-C-T-S-I-C-S-L-Y-Q-L-E-N-Y-C-N^{21}
B-chain F^{1}-V-N-Q-H-L-C-G-S-H-L-V-E-A-L-Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-T^{30}

Kyotorphin (bovine) : H-Tyr^{1}-Arg^{2}-OH

ß-LPH : E^{1}-L-T-G-Q-R-L-R-E-G-D-G-P-D-G-P-A-D-D-G-A-G-A-Q-A-D-L-E-H-S-L-L-V-A-A-E-K-K-D-E-G-P-Y-R-M-E-H-F-R-W-G-S-P-P-K-D-K-R-Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F-K-N-A-I-I-K-N-A-Y-K-K-G-E^{89}

Locust adipokinetic hormone (locust) : pGlu^{1}-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr^{10}-NH_2

LHRH : pGlu^{1}-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly^{10}-NH_2

α-MSH : Ac-Ser^{1}-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val^{13}-NH_2

γ_3-MSH : H-Tyr^{1}-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe^{11}-Gly-Arg-Arg-Asn-Ser-Ser-Ser-Ser-Gly-Ser-Ser-Gly-Ala-Gly-Gln^{26}-OH; γ_1-MSH is 1-11-NH_2

Motilin (pig) : H-Phe^{1}-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln^{22}-OH

α-Neoendorphin : H-Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys^{10}-OH; ß-neoendorphin is 1-9.

Neokyotorphin (bovine) : H-Thr^{1}-Ser-Lys-Tyr-Arg^{5}-OH

Neuromedin B-32 (pig) : H-Ala^{1}-Pro-Leu-Ser-Trp-Asp-Leu-Pro-Glu-Pro-Arg-Ser-Arg-Ala-Gly-Lys-Ile-Arg-Val-His-Pro-Arg-Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met^{32}-NH_2; neuromedin B-30 is 3-32; neuromedin B is 23-32.

Neuromedin U-25 (pig) : H-Phe^{1}-Lys-Val-Asp-Glu-Glu-Phe-Gln-Gly-Pro-Ile-Val-Ser-Gln-Asn-Arg-Arg-Tyr^{18}-Phe-Leu-Phe-Arg-Pro-Arg-Asn^{25}-NH_2; neuromedin U-8 is 18-25.

Neuropeptide K : H-Asp^{1}-Ala-Asp-Ser-Ser-Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His^{27}-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met^{36}-NH_2; substance K is 27-36.

NPY : H-Tyr^{1}-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr^{36}-NH_2

NT (bovine) : pGlu^{1}-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu^{13}-OH

ODN : H-Gln^{1}(or pGlu?)-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys^{18}-OH

OT : H-Cys^{1}-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly^{9}-NH_2

Prosomatostatin fragments (rat): A^{1}-P-S-D-P-R-L-R-Q-F-L-Q-K-S-L-A-A-A-A-G-K-Q-E-L-A-K-Y-F-L-A-E-L-L^{33}-S-E-P-N-Q-T-E-N-D-A-L-E-P-E-D-L-S-Q-A-A-E-Q-D-E-M-R-L-E-L-Q-R-S-A-N-S-N-P-A-M-A-P-R-E^{76}; the other fragment is 33-76.

Secretin : H-His^{1}-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Glu-Gly-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val^{27}-NH_2

SS : H-Ser^{1}-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala^{15}-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys^{28}-OH; SS-14 is 15-28.

SP (bovine) : H-Arg^{1}-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met^{11}-NH_2

TRH (rat) : pGlu^{1}-His-Pro^{3}-NH_2

VIP : H-His^{1}-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn^{28}-NH_2

VP : H-Cys^{1}-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly^{9}-NH_2 (AVP); LVP contains Lys.^{8}

a rather broad specificity (also known as enkephalinase); peptidyldipeptidase A; several aminopeptidases; and dipeptidylpeptidase IV.[72] Acetylcholinesterase can function as a peptidase for substance P and enkephalins but not for angiotensin II, bombesin, AVP or OT.[72] Other enzyme activities recently found in mammalian brain tissue, and involved in neuropeptide degradation, include a calcium-activated neutral protease from Japanese monkey brain,[86] aminopeptidase M,[87] a carboxypeptidase,[88] pyroglutamate aminopeptidase (which has a very narrow specificity)[89] and an isozyme of ACE.[90] The major aminopeptidase in human brain has recently been isolated.[91] This enzyme hydrolyzes enkephalins, AVP, OT, SS and CCK-8 (the sulfated form less well than the non-sulfated octapeptide) but not peptides with pGlu, arginine, or an acetyl group, at the N-terminus. Longer peptides, such as CRF and ß-endorphin, are not hydrolyzed to a significant extent, implying that the conformation of these peptides confers stability to hydrolysis. The partial purification of two distinct enkephalin-degrading aminopeptidases from human CSF was recently reported, but the physiological significance of such peptidases is still uncertain.[92]

Except for the longer-established neuropeptides, little information is available on biotransformation and degradation. Thus, proteolytic conversion with brain enzymes has been described for angiotensins,[72,86,93,94] bradykinin,[72,93,95] CCK,[72,74,91,96] dynorphins,[74,86,97] enkephalins,[72,74,86-88,91,93,95,98,99] LHRH[73-75,77,93,95] α- and ß-neo-endorphins[86,100] the neurohypophyseal hormones AVP and OT,[74,78,91,93,101] NT,[72,74,86,93,102] SS,[73-75,91,93] SP,[72,74,77,86,93,95,103] and TRH.[73-75,77,89,93,95] POMC-derived peptides are among the best studied. POMC may be cleaved at doublets of basic amino acids (Fig. 1) and at additional sites. For example, a chymotrypsin-like enzyme from porcine pituitary will cleave selected [Tyr-X] and [Phe-X] bonds in rat POMC and derived peptides.[104]

The degradation of ACTH has been studied extensively in peripheral tissues.[105,106] Endopeptidases are involved in the early stages. Comparatively few studies have been performed with brain enzymes. A study of the degradation of porcine ACTH by rat forebrain synaptic membranes showed that the fragment pattern was pH sensitive and that carboxypeptidase- and trypsin-like enzymes were involved[107] in the formation of smaller biologically active fragments.[108] When ACTH-(1-16)-NH_2 was incubated under similar conditions the principal products resulted from aminopeptidase-like activity.[109] The degradation of ACTH-(1-24) by mouse brain extracts was initiated by endopeptidase(s), though free amino acids were soon observed. The rate of release of amino acids from ACTH fragments is inversely related to fragment size.[105] Degradation studies of ACTH fragments (1-10), (1-4) and (7-10) with mouse brain extracts have also been reported.[105,110] Purified rat tonin showed trypsin/chymotrypsin-like activity in cleaving ACTH at specific Arg and Phe residues.[111] Incubation of α-MSH with rat brain homogenates results in rapid release of amino acids (except for Val-NH_2).[105] Synaptosomes from rat cerebral cortex are more selective: they cleave [Met-Glu] and [Phe-Arg] bonds.[112,113]

Proteolysis of ß-LPH and ß-endorphin by pituitary enzymes has been reviewed by Burbach.[35] It has been suggested that the conformation of the substrate directs selective enzymatic cleavage.[65] A number of preparations (e.g. calf brain cathepsin D) cleave the [Leu^{75}-Phe^{76}] bond in human ß-LPH and its equivalent in other species: further incubation may cleave the [Ala^{34}-Ala^{35}] bond.[35] Many additional

cleavage sites have been found in porcine ß-LPH, probably due to the (im)purity of the enzyme and/or the incubation conditions. Other enzymes which have been shown to give defined products are trypsin,[65] plasmin[35] and tonin.[111] Human brain ACE is strongly inhibited by ß-LPH.[114]

The potential physiological role of ß-endorphin, and its opiate-like properties, have prompted a large number of degradation studies.[35] Brain tissue homogenates (including human), soluble fractions, slice preparations, synaptosomes, synaptic membranes, CSF and purified enzymes have all been used to study the degradation of ß-endorphin, but only a few results are presented here. Cathepsin D[115] and another, unidentified, endopeptidase[116] each cleave the [Leu^{17}-Phe^{18}] bond to give γ-endorphin. Yet another endopeptidase cleaves the [Ala^{21}-Ile^{22}] bond. Exopeptidases are involved subsequently; for example, an aminopeptidase removes the terminal Tyr residue (but will not cleave the [Gly^{2}-Gly^{3}] bond) and carboxypeptidase(s) removes amino acids sequentially from the C-terminal end of γ-endorphin until [Pro^{13}] is reached.[117]

A single aminopeptidase from bovine brain selectively removes the N-terminal Tyr residue from α-, ß- and γ-endorphin.[118] *In vitro* studies with ß-endorphin in peripheral and brain tissue (including human putamen[119,120]) have confirmed that the proteolysis of ß-endorphin is a physiologically relevant pathway for the synthesis and regulation of other active peptides and not merely a degradation mechanism.[108] It has been suggested that ß-endorphin-(2-17) may have neuroleptic properties.[108] Treatment of this peptide with crude rat brain synaptosomal membranes[35] or with whole rat brain homogenates[121] gave ß-endorphin(6-17) as the major product.

Comment - The various processes involved in the formation and degradation of neuropeptides are potential targets for drug design. Progress to this end will be reviewed in the next volume of Annual Reports in Medicinal Chemistry.

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Section II - Pharmacodynamic Agents

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Chapter 7. Antihypertensive Agents

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Introduction - New understandings of the normal and/or pathologic modulators of blood pressure e.g. the renin angiotensin system (RAS),[1] atrial natriuretic factor (ANF),[2] calcium metabolism[3] and adrenoceptor mechanisms[4] continue to inspire new antihypertensive drugs. The beneficial impact of antihypertensive drug therapy on the decline of cardiovascular mortality is clear for stroke but is less well defined for other high risk patients.[5] The M.R.F.I.T. study has raised a warning flag concerning potential undesirable long term effects of thiazide diuretics especially in patients with existing arrhythmias.[6]

RAS Inhibitors - The RAS plays a critical role in the control of blood pressure and body fluid balance and is mediated primarily by the actions of angiotensin II (ANG II) generated by the conversion of the angiotensinogen substrate to the biologically inactive decapeptide ANG I by renin and then to the active octapeptide ANG II by angiotensin converting enzyme (ACE).[1] The efficacy of ACE inhibitors (ACEI) in the treatment of essential hypertension and congestive heart failure has lead to an intensified international search for new clinically useful RAS inhibitors.[7,8]

Renin Inhibitors - "Inhibitors" of renin have been recently reviewed.[9-11] Clinical trials of renin inhibitors have been limited, but both RIP (2) and H-142 (3) have been shown to lower arterial pressure in sodium depleted, normotensive volunteers.[12,13] The majority of preclinical data suggests that renin inhibitors and ACEI exert similar effects on arterial pressure.[10] The therapeutic advantage of renin inhibitors over the less selective ACEI (e.g. ACEI also affect kininase II) is unknown. Several chemical "strategies" have yielded extremely potent and specific renin inhibitors. H-261 (4) is a potent renin inhibitor based on the fully active N-terminal 13 or 14 amino acid sequence of angiotensinogen. In 4, the -Leu-Val- scissile bond has been replaced with a hydroxyethylene transition state mimic. Compound 4, 0.1 and 1.0 μg/kg/hr, reduced arterial pressure and plasma renin activity and circulating ANG II levels in anesthetized baboons, but all parameters rapidly returned to control levels when the infusion was stopped.[14] The central statine ((3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid) residue of pepstatin is also thought to function as a transition state analog.[15] From computer modeling of statine as the dipeptide replacement of the natural scissile bond, SCRIP (5) was proposed and shown to be a potent inhibitor of human renin.[16] The concept of a transition-state analog as a point of departure for designing novel

inhibitors has recently been reviewed.[17] Boger et. al. evaluated the susceptibility of statine containing compounds to inactivation by rat liver homogenates and found that substituting D-Pro for the natural L-Pro and benzylamide for the C-terminal Phe-NH_2 increased proteolytic stability.[18] However, the duration of action of 6 in Na^+-deficient dogs was not prolonged, likely due to rapid biliary excretion.[18] A series of peptides with modified -Phe-Phe- or statine cleavage sites has recently been reported.[19] The most potent compound 7 contains statine. R-Pep-27 (8), a nonapeptide containing statine, was reported to be a potent inhibitor of human renin in vitro.[20] Compound 8 i.v. transiently lowered blood pressure and plasma renin activity (PRA) in salt depleted but not salt replete conscious primates. Prolongation of in vivo activity of another statine containing nonapeptide, CGP29287 (9), was achieved by adding blocking groups to the terminal amino acids. The duration of 9 in the conscious marmoset ranged from 1 hr at 0.1 mg/kg to 3 hr at 10 mg/kg, i.v. Compound 9 is the first specific renin inhibitor

Compound #			Human Renin Inhibition[a]
		I. Angiotensinogen "Substrate" Analogs	
1.	Human	Asp...Ile-His-Pro-Phe-His-Leu-Val-Ile-His	2.9×10^{-5}
		A. Transition State or Cleavage Site Mimics	
2.	RIP	Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys	2×10^{-6}
3.	H-142	Pro-His-Pro-Phe-His-Phe-Leu^{R}-Val-Ile-His-Lys	1×10^{-8}
4.	H-261	Boc-His-Pro-Phe-His-Leu^{OH}-Val-Ile-His	0.7×10^{-9}
		B. Statine Containing	
5.	SCRIP	Iva-His-Pro-Phe-His-Sta--Leu-Phe-NH_2	1.6×10^{-8}
6.		Iva-His-(D)-Pro-Phe-His-Sta--Leu-$NHCH_2Ph$	1×10^{-8}
7.		Trp(in-Fm)Pro-Phe-His-Sta--Val-Trp(in-Fm)NH_2	3.8×10^{-10}
8.	R-Pep-27	Pro-His-Pro-His-Phe-His-Sta--Leu-Phe-Lys	2.0×10^{-6}
9.	CGP29287	Z-Arg-Arg-Pro-Phe-His-Sta--Ile-His-Lys(Boc)OMe	1×10^{-9}
10.	ES-305	BNMA-His-Sta--MBA	9.2×10^{-9}
11.		Boc-His-Pro-Phe-His-Sta--AHPHA	3.1×10^{-8}[b]
12.		Boc-Phe-Phe-Sta--Leu-m-AMBA	2.6×10^{-11}[b]
13.		Boc-Phe-His-NH-CH(i-Bu)$COCF_2CO$-Ile-NH_2CH_2-Pyr	1.4×10^{-9}
		C. Other Substrate Analogs	
14.		Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH_2	0.2×10^{-9}
15.	A-60956	Boc-Phe-His-AHBAPS	7.0×10^{-9}
16.		Boc-Phe-His-Leu(AA)-Gly-Ile-His-OMe	3.5×10^{-8}
		II. Pepstatin Analogs	
17.	Pepstatin A	Iva-Val-Val-Sta--Ala-Sta-OH	1.3×10^{-5}
18.	SR42128	Iva-Phe-Nle-Sta--Ala-Sta-OH	2.8×10^{-8}
		III. Prosegment Fragments	
19.		Boc-Leu-Lys-Arg-Met-Pro-Sta-OH	3×10^{-4}
		IV. Other	
20.		Et-Phe-(D)-Met-Gly-Phe-NH2Ad	9×10^{-5}
21.		Boc-Phe-Leu-V	8.7×10^{-6}

[a] IC_{50} or K_i against human plasma renin (M); [b] K_i against human kidney renin; R = reduced peptide bond ($-CH_2NH-$); OH = hydroxyethylene ($-CHOHCH_2-$); m-AMBA = m-aminomethylbenzylamide; AA = amino alcohol; NH2Ad -2-aminoadamantane; V = 1-benzyl-(2,3-dihydroxypropyl)amino; AHBAPS = 3-amino-2-hydroxy-4-cyclohexylbutyl 4-methyl-2-(dihydrocinnamoylamino)-pentyl sulfide; ACHPA = (3S,4S)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid; BNMA = bis(1-naphthyl)methylacetyl; AHPHA = (2S,3S,4S)-4-amino-3-hydroxy-2-phenylmethyl-7-methylheptano amide; MBA = 2-(S)-methylbutylamide

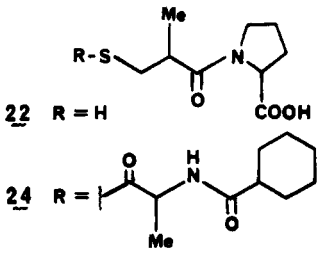
reported to have oral activity even though the hypotensive effect of a high dose (100 mg/kg) lasted less than 120 min.[21,22] High renin inhibitory potency *in vitro* has been achieved with several new series of statine containing compounds that have reduced molecular size and number of peptide bonds. A series of histidyl-statine analogs has been described with the most potent being ES-305 (10).[23] The *in vitro* inhibitory effect of 10 is similar for human and monkey renin and is much less against renin from other species, or against other proteases. A search for shorter peptides containing nonpeptidal segments and with enhanced duration of action and oral activity led to the discovery of potent new statine containing "dipeptides" (e.g. compound 11)[24] and "pentapeptide" (e.g. compound 12)[25] analogs. The K_i for 12 against purified human kidney renin is 0.026 nM, whereas the IC_{50} against human plasma renin is > 1 μM.[25] This dramatic difference in renin inhibitory potency is apparently due to the binding of 12 to plasma proteins. The fluoro statinone derivative 13 exhibited enhanced potency over the desfluoro analog.[26] Replacement of statine with (3S,4S)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid has yielded even more potent renin inhibitors.[27] One of these compounds, 14, is 55-76 times as potent as the comparable statine containing compound against human renin *in vitro* and 19 times as potent in lowering pressure in Na^+-deficient dogs.[27] A-60956 (15), an additional substrate analog, has a similar renin inhibitory profile *in vitro*.[28] A new series of renin inhibitors incorporated an amino alcohol function as a non-hydrolyzable substitute for the scissile bond of angiotensinogen.[29] Compound 16 is the most active of these compounds and is much less potent against hog kidney than human plasma renin (IC_{50} 1200 versus 3.5 nM). It is virtually inactive against pepsin or cathepsin D.

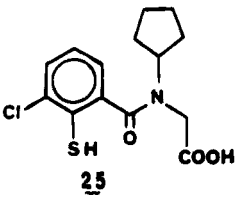
Pepstatin was originally derived from actinomycetes and contains two statine residues.[30] Pepstatin A (17) lacks the affinity and specificity for human renin to be a useful therapeutic agent, however, substitution of the -Val-Val- constituents of pepstatin with other amino acids, e.g. Phe-Nle, has yielded markedly more potent agents.[31] SR42128 (18) shows lesser species specificity for renin inhibition (IC_{50}s for man, rat and dogs: 2.8×10^{-8}M, 6×10^{-7}M and 4.8×10^{-8}M, respectively). Compound 18, 1-10 mg/kg, i.v., produced a dose-related decrease in arterial pressure in Na^+-deplete monkeys with a duration of action >3 hours, suggesting that inhibitors with 2 statine residues are more resistant to proteolytic cleavage.[31]

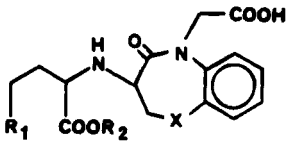
The deduction of the preprorenin precursor of human renin from the structural gene coding for human renal renin[32] has led to the synthesis and testing of a number of incomplete segments (prosegments).[33] Analogous to previous studies which have shown that prosegments of mouse submaxillary renin inhibited the intact enzyme,[34] a number of peptide "prosegments" (e.g. 19) inhibited human plasma renin, especially those including the NH_2-terminal portion.[33] A number of other approaches to the discovery/design of renin inhibitors have been reported. Compound 20, 3 mg/kg, i.v., demonstrated a brief (<20 min) hypotensive effect in Na^+-deficient rhesus monkeys.[35] Compound 21, a "dipeptide glycol", 10 mg/kg, i.v., briefly lowered PRA in rhesus monkeys.[36]

ACE Inhibitors - The chemistry and pharmacology of ACEI have been reviewed.[37-39] Recent clinical developments include a long-term multicenter surveillance sudy of captopril (22)[40] and examination of enalapril (23) in patients with heart failure.[41] The captopril analog 24 (MC-838) in normal volunteers reduced blood pressure with a longer duration of action than 22.[42] The mercaptoaroyl compound 25 was ten

times less potent in vitro than 22, yet was orally active in spontaneously hypertensive rats (SHR).[43] Benzazepin-2-one 26 exhibited a similar biological profile compared to 23 in the dog[44] and 27 (CGS 14824A) paralleled 23 in the SHR.[45] The benzothiazine 28 was equally potent to 27 when compared i.v. in the SHR, but was less efficacious when administered orally.[46] The oral potency of 29 was similar to 23 in the dog.[47] The replacement of the amino moiety of CI-906 (30) with an oxygen atom provided 31, which showed modest oral antihypertensive

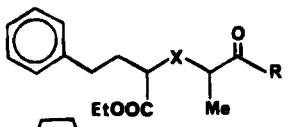


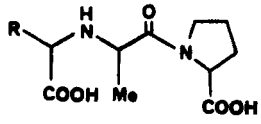


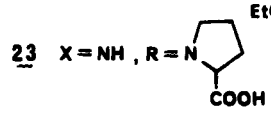


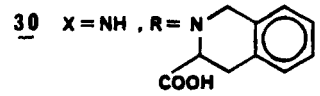
	R_1	R_2	X
26	$(CH_2)_2NH_2$	H	CH_2
27	Ph	Et	CH_2
28	Ph	Et	S

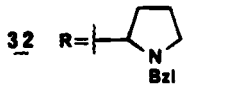
activity in the renal hypertensive rat.[48] Acyl tripeptide analogues of enalaprilat were reported. Since 32 and 33 furnished in vitro inhibitory potencies equal to enalaprilat, the extended peptide chains are not contributors to additional binding interactions.[49] Pentapeptides 34 and 35 were approximately 30-40 times more potent than 22 in vitro but

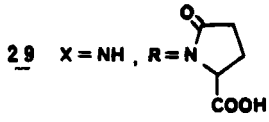


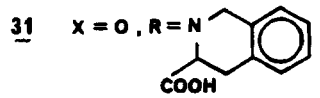


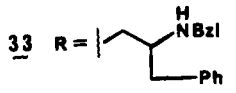




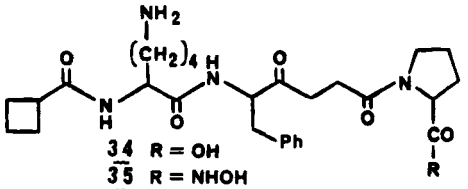


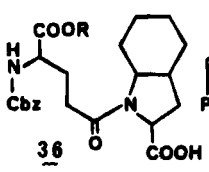


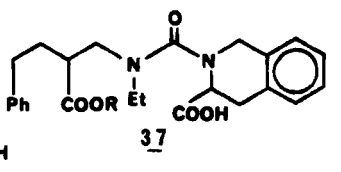




did not lower blood pressure in renal hypertensive rats.[50,51] A tritiated derivative of 34 revealed that 70% of an oral dose was absorbed and rapidly excreted unchanged from the blood with a half life of 24 min.[51] Compound 36 (R=H) was a potent in vitro ACEI, and although its prodrug 36 (R=Et) inhibited the ANG I response in normotensive rats when administered i.v. it displayed no oral efficacy.[52] A carboxyethylcarbamoyl derivative 37 (R=H) was comparable to 22 in vitro and the ester 37 (R=Et) similiarly lowered blood pressure in the SHR when administered orally.[53] The fluoro ketone group in 38 increased ACE inhibition in vitro by 300-fold over methyl ketone 39.[54] The utilization of

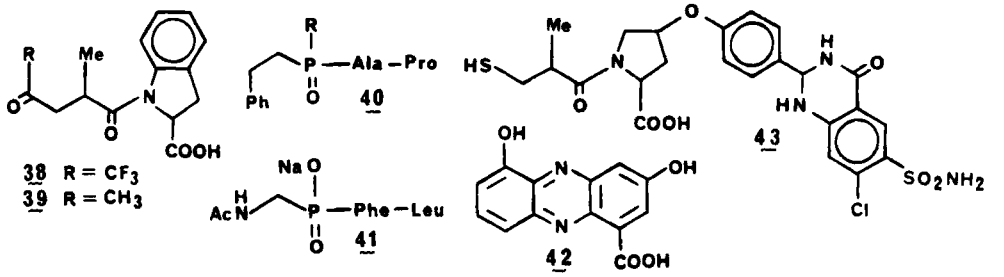






phosphonamidates as tetrahedral species furnished 40 (R=OPh).[55] Nonenzymatic hydrolysis of the phenyl ester gave 40 (R=OH) which was a potent in vitro ACEI. Phosphonamidate 41 was several orders of magnitude

less potent against ACE compared with enalaprilat.[56] Evaluation of fermentation-derived molecules as ACEI revealed phenacein 42[57] and ancovenin[58] with in vitro potencies of approximately ten and 150-fold, respectively, less than 22. The interruption of the RAS utilizing bradykinin-derived substrates furnished the des-Pro derivative Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg, which was seven times more potent in vitro than 22 against ACE.[59] A bradykinin potentiating peptide (BPP_{5a}) analog, [gPhe^3,(R,S)-mAla^4]BPP_{5a}, possessing an inverted amide bond, was a moderate ACEI in vitro and was more potent than BPP_{5a} as a hypotensive in normotensive rats.[60] The synergistic and beneficial therapeutic effects of the combination of an ACEI and diuretic have been reported.[61] The incorporation of 22 and a diuretic through a nonlabile linkage was described.[62] Compound 43 (SQ27,786) completely inhibited the ANG I pressor response in conscious normotensive dogs when administered i.v. Although urine volume and sodium excretion were increased by 43, blood pressure was not affected.

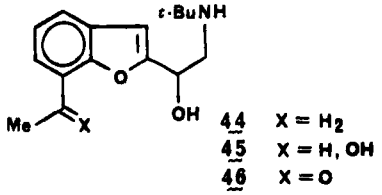


Atrial Peptides (APs) - With most of the structural identities of the natriuretic and vasorelaxant peptides secured, an increased understanding of their role and mechanism is being vigorously pursued. Several comprehensive reviews have appeared.[63-65] ANF (106-125), which lacks the intramolecular cystine bridge, was two to three orders of magnitude less potent in the smooth muscle relaxant activity assays of rabbit aorta and chick rectum than the peptide embodied with the disulfide linkage, ANF (103-125).[66] A SAR evaluation of the length of ANF with the maximal biological activities was described.[67] N-terminal amino acid extension on APIII (Ser-Leu-Arg-Arg-APIII) exhibited enhanced natriuretic-diuretic effectiveness, but no difference in renal blood flow in the dog.[68]

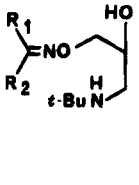
Calcium Antagonists - The primary role of calcium in excitation-contraction coupling of vascular smooth muscle and its possible involvement in the etiology of hypertension[3,69] continues to stimulate new drug discovery and development (see Chapter 9).

ß-Adrenoceptor Blocking Agents - Reports on the therapeutic utilization of ß-blockers in myocardial infarction,[70] concerns for asthmatic patients,[71,72] and adverse effects[73] have appeared. Comparisons of ß-blocking agents with respect to renal function,[74] antihypertensive potencies,[75] blood pressure during mental and physical activity,[76] and the circadian rhythm of hypertension[77] have been reported. Patients treated with ß-blockers had a 50% increase in the density of ß-adrenergic receptors in the plasma membranes of right atrial tissue removed during cardiac bypass surgery.[78] The ß-adrenoceptor activities of the stereoisomers of bufuralol (44) and metabolites 45 and 46 were examined.[79] The anti-isomer of falintolol (47) is seven times more potent than the syn-isomer on the guinea pig trachea. In atria, the

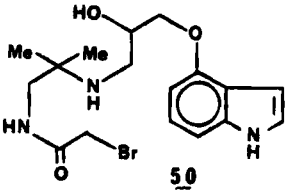
anti-isomer is four times more effective than the syn-isomer. The dicyclopropyl derivative 48 is twice as potent on atria as 47.[80] Models of compound 49 and toliprolol were evaluated by electrostatic molecular potential and correlated with receptor binding.[81] Compound 50, a potent, irreversible ß-adrenergic derivative of pindolol, was devoid of agonist properties.[82]



44 X = H_2
45 X = H, OH
46 X = O

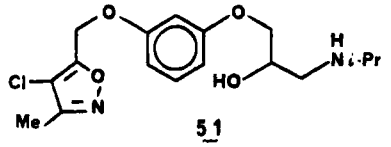


	R_1	R_2
47	△	Me
48	△	△
49	Me	Me



50

OF-4452 (51) exhibited greater ß-antagonist activities in rats and in isolated guinea pig heart atria than propranolol.[83] CGS-10,078 (52) decreased the cardiac index in Wistar-Kyoto rats, due to its ß-antagonist effects, and produced no change in total peripheral resistance index because of its combined direct vasodilation from α-adrenergic antagonism and slow calcium channel blockade.[84] SC-36859 (53), a ß-adrenergic agonist, reduced total peripheral resistance and was a long acting orally active antihypertensive in the SHR.[85]



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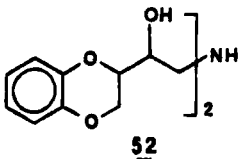

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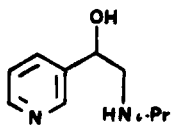

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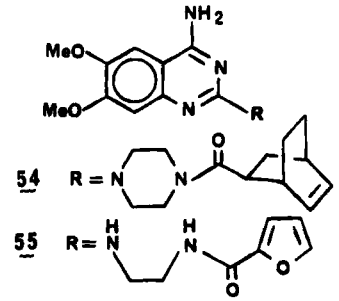
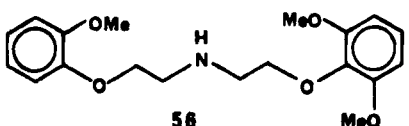
α-Adrenoceptor Agonists and Antagonists - The functions of peripheral α-adrenergic agents[86] and central α-agonists[87] in heart failure and the consequences of α_2-agonists on renal function[88] and their treatment of hypertension[89,90] have been reviewed. α-Receptor blocking activities in rat vas deferens were exhibited by two derivatives of prazosin: compounds 54 (SM2470)[91] and 55 (α_1/α_2 > 3500).[92] ACC-7513 (56) had twenty times more affinity for α-receptors than for 5-HT-receptors in rabbit aortic strips.[93] Dual blocking activity (α/β_1 = 1) was shown for 57.[94] Compound 58, an analog of the α_2-adrenoceptor antagonist RX 781094 (59),[95,96] was eight times more



54 R = N

55 R = N



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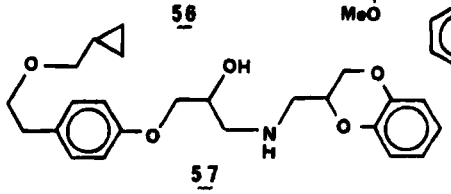

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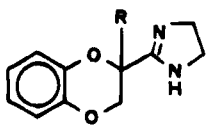
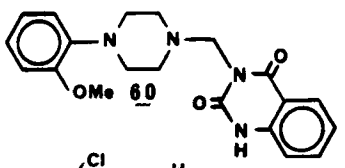
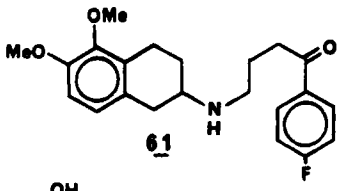
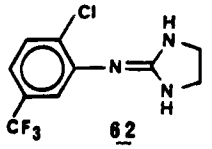
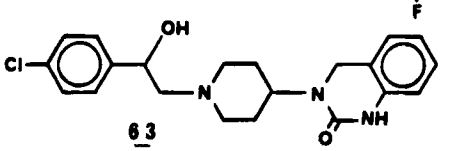


58 R = OMe
59 R = H



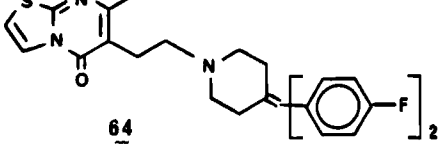


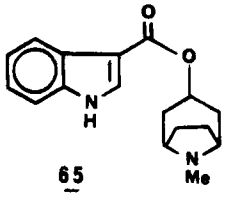




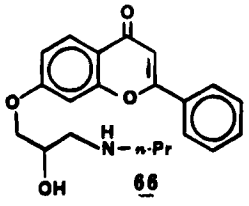
potent than 59 and the α_2/α_1 selectivity ratio was two-fold greater.[97] SGB1534 (60) displayed potent α_1-receptor blocking activity in the dog.[98] Ligand binding studies of rat cerebral cortex revealed aminotetralin 61 possessed moderate α_1 affinity.[99] St-587 (62) displayed α_1-agonist and α_2-antagonist properties in anesthetized dogs.[100] Compound 63 (KF5908) exhibited α-receptor affinities on rat thoracic aorta but not on vas deferens.[101]

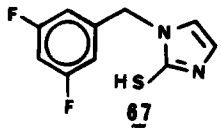
Serotonin (5-HT) Antagonists - The involvement of 5-HT in the cardiovascular system and the blood pressure lowering effects of antagonists have been reviewed.[102,103] Other 5-HT_2 antagonists are being evaluated for additional indications e.g. mood elevation (ritanserin, 64);[104] antiarrhythmic (ICS 205-930, 65).[105]

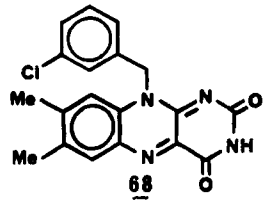




Vasodilators - Decreasing vascular resistance with vasodilators alone or in combination is a rational and useful treatment of hypertension[106] and new compounds with differing mechanisms of action continue to be described. At antihypertensive doses in the SHR, flavodilol (66) reduces catecholamines in the heart to a much greater extent than in the brain.[107] The fall in norepinephrine content of mesenteric arteries and brain (and dopamine in mesenteric arteries) correlated with the antihypertensive effects of SKF102698 (67), a new dopamine-beta hydroxylase inhibitor, in SHR.[108] The riboflavin analog L-632481 (68) was shown to lower blood pressure in SHR and suppress hydrochlorothaizide-induced hyperaldosteronism.[109]







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Chapter 8. Pulmonary and Antiallergy Agents

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INTRODUCTION - Medicinal chemical research in pulmonary and allergic diseases continues to focus on the role of leukotrienes (LTs) as major causative agents in bronchoconstriction, edema, and inflammation. Several potent orally active receptor antagonists and biosynthesis inhibitors have emerged as potential clinical candidates. In addition to leukotriene research, interest continues in selective phospholipase A_2 inhibitors, non-sedating antihistamines, and phosphodiesterase inhibitors.

LEUKOTRIENES

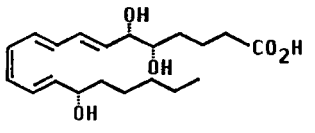
Pharmacology and Biochemistry of Leukotrienes - Throughout the year, evidence emerged that further supports the proposed role of LTs as key mediators of a diverse array of inflammatory states and immunologic diseases. Several comprehensive reviews encompassing the biosynthesis, metabolism, pharmacology, pathophysiology, and clinical implications of the LTs were published.[1-6] Although the hypothesis that the LTs are major mediators of reversible obstructive airways disease (asthma) continues to await clinical verification, several pieces of circumstantial evidence appeared which support this proposal. LTC_4, LTD_4, and LTE_4 were detected in the plasma,[7-9] sputum,[10] and bronchial lavages[8] of individuals with allergic asthma in concentrations which correlate directly with the severity of the disease.[7,9,10] Peptido-LTs were also detected in the serum of patients during exercise-induced asthma.[11] LTB_4 was present in sputum[10] and plasma[12] of subjects with active asthma, but not in well-controlled asthmatics[10] or normal volunteers.[12] Asthmatic patients displayed marked airway hyperreactivity, a hallmark of bronchial asthma, to inhaled LTD_4.[13,14] Specific LTC_4 [15] and LTD_4 [16] binding sites were identified in human lung parenchyma. Evidence directly supporting a role of the LTs in antigen-induced bronchospasm was provided by experiments with passively sensitized human bronchi, where the selective LT receptor antagonist SK&F 102922 partially inhibited the contractile response to anti-IgE, and a combination of SK&F 102922 and mepyramine (a histamine H_1 receptor antagonist) abolished the contractile response.[17] The vital piece of information not yet available is whether a potent and specific LT antagonist or LT synthesis inhibitor will ameliorate asthmatic episodes.

Evidence of LT involvement in respiratory disorders other than bronchial asthma also appeared. LTB_4, LTC_4, LTD_4, and LTE_4 were detected in nasal washings of patients with allergic rhinitis following nasal challenges with a specific allergen.[18] LTB_4 and LTC_4 were detected in serum samples of patients with cystic fibrosis, bronchiectasis, and chronic bronchitis.[19]

Previous studies suggesting the existence of separate receptors for LTC_4 and LTD_4 were confirmed using both pharmacologic and biochemical approaches.[20,21] Contractile responses of guinea pig airway to various

LTs and LT analogs correlate well with radioligand competition curves for the $[^3H]$-LTD_4 binding site,[20,21] but not for the $[^3H]$-LTC_4 binding site.[20] Furthermore, unlike $[^3H]$-LTD_4 binding sites, which had a limited tissue distribution, $[^3H]$-LTC_4 binding sites were nearly ubiquitous.[20,21] In another study, it was found that LTD_4 and LTE_4 bind to the same receptor.[22] The ability of guanine nucleotides to regulate the binding of $[^3H]$-LTD_4/E_4 to this receptor prompted the suggestion that it is coupled to a guanine nucleotide regulatory protein.[22]

Synthesis of Leukotrienes and Related Compounds - An excellent comprehensive review on the chemistry of the prostaglandins and leukotrienes has appeared,[23] as has a more narrowly focused review on the synthesis of leukotrienes and lipoxygenase products.[24] The recently discovered lipoxins, arachidonic acid metabolites that stimulate superoxide generation and degranulation of neutrophils, have been synthesized by several methods, and stereochemical assignments have been made. Lipoxin A (1) has been prepared from 15(S)-HETE[25] and LTA_4,[26] as well as by total synthesis.[26,27] Lipoxin B consists of major and minor components, but there have been conflicting reports as to their identity. One group claims them to be structures 2 and 3, respectively,[28] whereas a second group reports them to be 4 and 5.[29,30] All four of these isomers have been prepared by a single synthetic strategy, utilizing a Pd-Cu catalysed coupling reaction as the key step.[31] Recent studies support the assignment of structures 4 and 5 for lipoxin B, and furthermore suggest that lipoxin A itself consists of four isomers.[32] LTB_4 has been obtained through the photolysis of a chiral bicyclic ketone.[33] Iso-LTB_4 (5,13-dihydroxy),[34] LTB_3,[35] and the 7Z,9E,11Z,14E isomers of LTC_4, LTD_4, and LTE_4[36] have all been prepared by more conventional methods.



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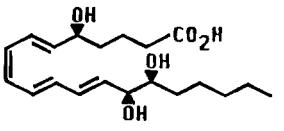

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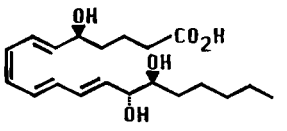

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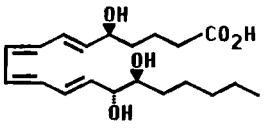

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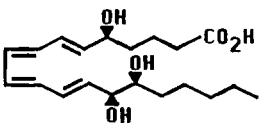

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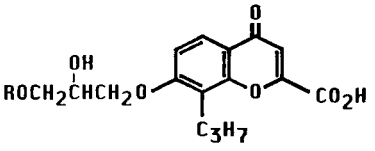
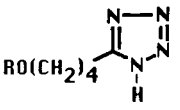
Leukotriene Receptor Antagonists - A number of potent peptidoleukotriene receptor antagonists have been reported, with several compounds advancing to early clinical trial. Most of the antagonists are structurally related, and comparable in potency, to the initial lead, FPL-55712, but in addition possess significant oral efficacy. LY-171883 (6) is a competitive and selective antagonist of LTD_4 on guinea pig ileum and lung parenchyma (pK_B = 6.5). It is non-competitive against LTD_4 on guinea pig trachea and 50-fold less potent against LTC_4.[37] *In vivo* (10 mg/kg, p.o.), it blocks bronchoconstriction elicited by either LTD_4 or antigen. It is also a potent phosphodiesterase inhibitor and may have bronchodilator activity (IC_{50} = 22.6 μM for inhibition of human PMN phosphodiesterase). It produces reversible hepatomegaly in rodents, an effect which is absent in guinea pigs, dogs, and monkeys.[38] A related analog, LY-163443 (7), has a similar profile but is more potent (pK_B = 8.1, 7.5, and 7.5 on guinea pig ileum, trachea, and parenchyma,

respectively).[39] Another compound, L-649,923 (8), is a competitive antagonist of LTD_4 on guinea pig ileum (pK_B = 8.1) and of LTE_4 on guinea pig trachea (pK_B = 7.1), but non-competitive vs. LTD_4 on guinea pig trachea.[40] In rats it blocks bronchoconstriction induced by LTD_4 (ED_{50} = 0.26 mg/kg, i.v.) and antigen (ED_{50} = 1.5 mg/kg, p.o.), and in squirrel monkeys inhibits LTD_4-induced bronchoconstriction (5-10 mg/kg, p.o.). It is reportedly undergoing clinical evaluation.[40] A fourth structurally related compound, Ro-23-3544 (9), is an antagonist of LTD_4-induced bronchoconstriction in guinea pigs and is 80-fold more potent than FPL-55712 by the aerosol route.[41] Although inactive against histamine- and PAF-induced bronchoconstriction, it inhibits LTB_4-induced lung bronchoconstriction.



FPL-55712



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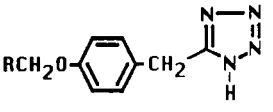

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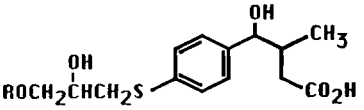

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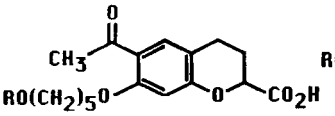

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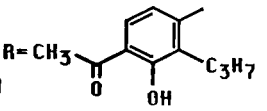
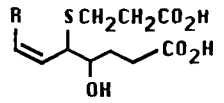
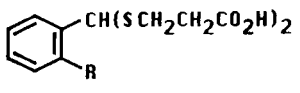


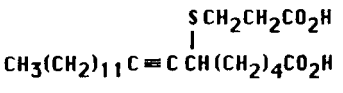
All of the above compounds are structural variants of FPL-55712 and tend to share a common pharmacological profile. However, several compounds have been prepared which are structurally derived from the natural agonist, LTD_4. A series of 2-nor-leukotriene analogs, as exemplified by 10a, are antagonists of LTD_4-induced contractions of guinea pig trachea (pK_B = 5.5-6.5).[42] Potency in this series is highly dependent upon the polar functional groups.[43] The length of the alkyl chain is also critical; subtraction (10b) or addition (10c) of two methylene residues significantly reduces activity.[42] This suggests that there is a structural requirement for the hydrocarbon moiety and that it is not merely serving a lipophilic function. Two dithioacetals, 11 and 12 (SK&F 102081 and 102922), have been described.[44] Compound 12 is a selective and competitive LTD_4 antagonist in guinea pig trachea (pK_B = 6.7), comparable to FPL-55712. However, it exhibits ten-fold higher affinity for LTD_4 binding sites in guinea pig lung membranes (K_i = 245 nM vs. 2.2 μM for FPL-55712). In addition to enhanced potency, the phenyloctyl group of 12 provides resistance to ω-oxidative metabolism in vivo.[45,46] Aerosolized 11 and 12 afford good protection against LTD_4-induced bronchospasm in guinea pigs. The 7,8-acetylenic analog (13) and several related compounds antagonize the LTC_4-induced



10a, R = $CH_3(CH_2)_{11}$
10b, R = $CH_3(CH_2)_9$
10c, R = $CH_3(CH_2)_{13}$



11, R = $(CH_2)_{11}CH_3$
12, R = $(CH_2)_8Ph$



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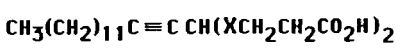
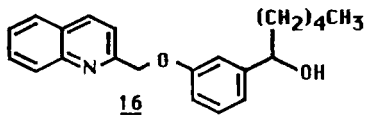


14, X = O
15, X = S

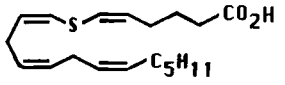


16

contractions of isolated guinea pig lung strips.[47] Similarly, 14 and 15 display marked LTC_4 antagonist activity. This inhibition could be a consequence of either LTC_4 receptor blockade or inhibition of metabolically-generated LTD_4.[48] The LT biosynthesis inhibitor, REV 5901 (16), competitively antagonizes LTD_4-induced contractions of guinea pig lung parenchymal strips (IC_{50} = 2 μM).[49]

Leukotriene Biosynthesis - Considerable progress has been made in the characterization and purification of the enzymes involved in leukotriene biosynthesis. Arachidonate 5-lipoxygenase (5-LO) has been purified from homogenates of human peripheral blood leukocytes.[50] Gel electrophoresis of the purified protein revealed a single major band (apparent M_r 80,000). A membrane-associated 5-LO stimulatory factor which may play a role in the regulation of this enzyme was characterized.[51] The 5-LO and LTA_4-synthetase enzymatic activities from human leukocytes,[52] porcine leukocytes,[53] and potato tubers[54] co-purify in a single protein, indicative of a dual-function enzyme. In contrast, 5-LO purified from RBL-1 cells (single protein band, M_r 73,000 on SDS gel) appears devoid of LTA_4-synthetase activity.[55] LTA_4-hydrolase has been purified from a variety of sources, including human erythrocytes[56] and human[57] and rat neutrophils.[58] LTA_3 is a poor substrate but a potent inhibitor of this enzyme;[59] LTA_5 behaves similarily.[60] A role for extracellular LTA_4 in the production of LTC_4 has been demonstrated in murine mast cells. LTA_4, a product released by stimulated PMNLs, is efficiently metabolized to LTC_4 by resting mast cells. Thus, cooperativity between cells may be important in the biosynthesis of the peptidoleukotrienes.[61]

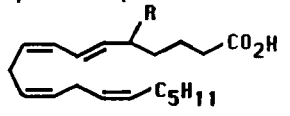
Inhibition of Leukotriene Biosynthesis - The discovery of effective and selective inhibitors of leukotriene biosynthesis is currently under investigation by a number of groups. The predominant target enzyme is 5-LO, although inhibitors of LTA_4-hydrolase and glutathione-epoxide transferase have been reported. An excellent review on inhibitors of leukotriene biosynthesis has appeared.[62] Several groups have explored substrate analogs as competitive or k_{cat} inhibitors of 5-LO. 7-Thiaarachidonate (17), as well as a number of chain shortened cis,cis-divinylsulfides (18), are oxygen- and time-dependent irreversible inhibitors of the 5-LO of RBL-1 cells.[63] The sulfoxides of 17 and 18 are reversible competitive inhibitors. Replacement of the carboxylate function of 18a with a hydroxamate (18b) changes the kinetics of inhibition from time-dependent to competitive (K_i = 4.2 μM).



17

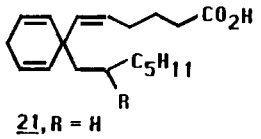
18a, X = OH

18b, X = NHOH



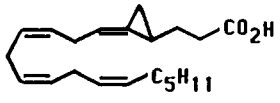
19, R = CONHOH

20, R = $CH_2CONHOH$



21, R = H

22, R = OH



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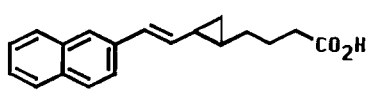

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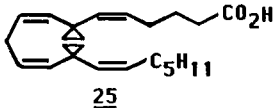
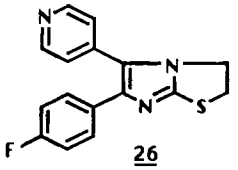


25

Presumably, the hydroxamate function chelates the prosthetic iron and prevents the catalytic function essential for K_{cat} inhibition.[62] Positioning a hydroxamic acid-containing group at carbon-5 of AA, a position presumably in close proximity to the iron atom in the enzyme, provides 19 and 20, potent inhibitors of 5-LO (IC_{50} = 1.4 μM, 0.19 μM, respectively).[64] The spiro-triene analog (21) of arachidonic acid is a modest inhibitor of 5-LO (IC_{50} = 120 μM). Introduction of a hydroxyl group, suggested by a report that 15-HETE is a 5-LO inhibitor,[65] affords the potent and selective inhibitor 22 (IC_{50} = 6 μM for 5-LO *vs.* > 750 μM for prostaglandin synthetase).[66] The synthesis of the methylenecyclopropane analog (23) has been reported, but no data were disclosed.[67] The cyclopropyl compound 24 (TEI-8005) inhibits both 5-LO and 12-LO in the 10-50 μM range.[68] A series of mono-, di-, and triethanoarachidonic acids failed to inhibit LTC_4 and LTD_4 production in cat lung tissue, although 7,13-diethanoarachidonic acid (25) significantly inhibited LTD_4-induced cat coronary artery constriction (43% inhibition at 5 μM).[69]

A variety of structurally diverse 5-lipoxygenase inhibitors has been reported. SK&F 86002 (26) is an orally active dual 5-lipoxygenase/cyclooxygenase (CO) inhibitor (IC_{50} = 7.5 μM *vs.* 5-LO from RBL-1 cells), and has potent anti-inflammatory effects in animal models generally insensitive to CO inhibitors (ED_{50} = 50 mg/kg, p.o. in arachidonic acid-induced edema in mouse ear and rat paw).[70] Another compound, L-652,343 (27), inhibits leukotriene and prostanoid synthesis at sub-micromolar levels (IC_{50} = 0.5 μM for ram seminal vesicle CO; IC_{50} = 2 μM *vs.* 5-LO from RBL-1 cells). The structural requirements for the observed activity of 27 have been described. The 5-LO activity results primarily from the 3-hydroxybenzothiophene substructure, whereas the enamide side chain is necessary for potent CO inhibition.[71] The anti-oxidant trihydroxyisoflavan 28 exhibits potent inhibition of human PMNL 5-LO (IC_{50} = 1.2 μM) with significantly less activity against CO (IC_{50} = 200 μM) and human platelet 12-LO (IC_{50} = 22 μM).[72] Diphenyl disulfide reversibly inhibits RBL-1 5-LO at 1-5 μM.[73] Gossypol (29) is a potent and specific inhibitor of RBL-1 5-LO and 12-LO enzymes (IC_{50} = 0.3 μM and 0.7 μM, respectively).[74] A novel seleno-organic compound, ebselen (30, PZ-51), inhibits the production of 5-HETE and LTB_4 by stimulated rat PMNL cells.[75] Several stilbenes isolated from the roots of Polygonum species, plants which have been used for the treatment of allergic and inflammatory diseases, inhibit rat PMNL 5-LO and CO enzymes at sub-micromolar concentrations.[76]



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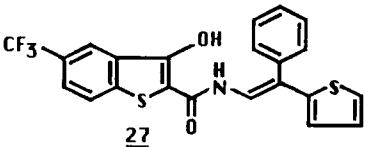

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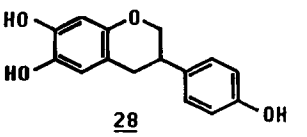

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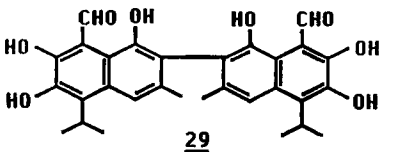

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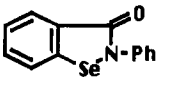

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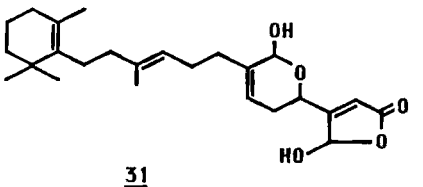
PHOSPHOLIPASES - In contrast to earlier reports, D-dihexanoylphosphatidylcholine was found to be a partial competitive inhibitor of the PLA_2 hydrolysis of the L-isomer.[77] Human platelet PLA_2 is inhibited by a number of unsaturated fatty acids, including palmitoleic, oleic, linoleic, linolenic, and arachidonic, with IC_{50}s of *ca*. 0.5 μM. This inhibition is apparently noncompetitive.[78] This enzyme is also inhibited by 5-, 12-, and 15-HETE, with IC_{50} values of 42, 26, and 72μM, respectively.[79] The marine natural product manoalide (31) is a potent PLA_2 inhibitor, irreversibly reacting with lysine residues.[80] The fungal metabolites plastatin (32) and luteosporin (33) inhibit pancreatic PLA_2 with K_i values of 0.89 and 12.8 μM, respectively.[81,82]

The anti-malarial drugs chloroquine and mepacrine, at 10-100 μM, inhibit leukotriene release from unsensitized human lung and sensitized guinea pig lung. These effects are countered by the presence of arachidonate, suggesting an effect on PLA_2.[83] Mepacrine also inhibits ischemia-induced PLA_2 activation in porcine myocardium.[84] The lipoxygenase/cyclooxygenase inhibitors nordihydroguaiaretic acid, 5,8,11,14-eicosatetraynoic acid, and quercetin inhibit PLA_2 activity of neutrophil extracts and sonicates, with IC_{50}s in the 10-100 μM range.[85] The non-steroidal anti-inflammatory drug tiaramide (34) inhibits rabbit platelet aggregation induced by collagen, but not by arachidonate.[86] Patents claiming PLA_2 inhibitory activity for compounds such as 35[87] and 36[88] have appeared. Structurally undefined PLA_2 inhibitory factors have been located in bovine plasma[89] and human amniotic fluid.[90]



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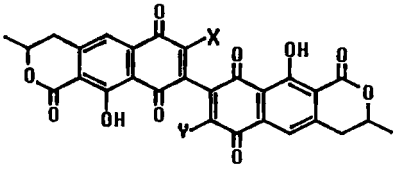
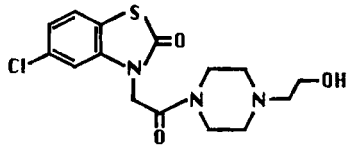


32, X = NH_2, Y = OH

33, X = Y = OH



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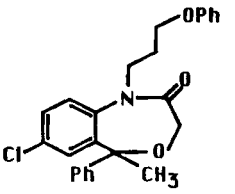

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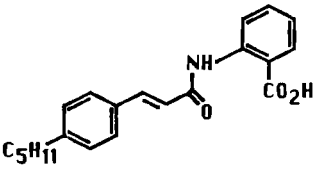

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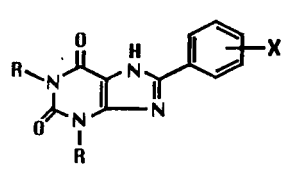
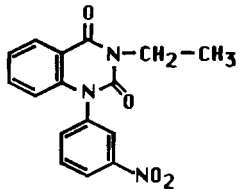
A review article describes the PLA_2-inhibitory action of the glucocorticoids.[91] These steroids exert their anti-inflammatory action *via* the production of macrocortin (lipocortin), an endogenous inhibitor of PLA_2. In rat peritoneal leukocytes, both hydrocortisone and macrocortin inhibit the release of the PAF precursor, 2-lyso-PAF. This inhibition could comprise an important component of their anti-anaphylactic and anti-inflammatory action.[92] Dexamethasone inhibits carrageenin-induced edema in the rat paw, a standard model of experimental inflammation that involves arachidonate metabolites. To a lesser extent, this steroid also inhibits dextran-induced edema (mediated largely by histamine and 5-hydroxytryptamine), apparently through the generation of other regulatory proteins.[93] Dexamethasone also has a direct effect on snake venom PLA_2, manifesting its inhibition within 3-10 minutes.[94]

XANTHINES AND PHOSPHODIESTERASE INHIBITORS - The pharmacodynamics of theophylline as an antiasthmatic were reviewed.[95,96] The original view that the bronchodilatory action of theophylline is attributable solely to an inhibition of cyclic nucleotide phosphodiesterase activity has become untenable and alternative mechanisms were proposed.[95,96] One of these alternatives, that theophylline acts as an adenosine receptor antagonist, was supported by a study indicating that theophylline preferentially inhibits adenosine-induced bronchoconstriction in asthmatic subjects.[97] Clearly, much more evidence is needed before the "adenosine hypothesis" can be accepted. One approach to resolving this issue is to develop selective, high affinity adenosine receptor antagonists for clinical evaluation. Steps toward this process were taken by analyzing a series of 8-phenylxanthines (37) for adenosine A_1-receptor antagonist activity.[98] These studies revealed the most potent A_1-receptor antagonists to be the 1,3-disubstituted n-propyl compounds, which have IC_{50}s in receptor binding assays and physiologic effects at concentrations 2-3 orders of magnitude below those of theophylline.[98] Sulfonamide substitution at the para phenyl position had little effect on potency but greatly increased the aqueous solubility of these compounds.[98] In another study, the relative potency of various 8-phenylxanthines at adenosine A_1-receptors vs. A_2-receptors was determined.[99] 1,3-Dipropyl-8-phenylxanthine was somewhat selective (23-fold) for A_1-receptors over A_2-receptors, whereas 8-(2-amino-4-chorophenyl)-1,3-dipropylxanthine was nearly 400-fold more potent at the A_1-receptor.[99] The water-soluble 8-(p-sulfophenyl)-1,3-dipropylxanthine derivatives no longer possessed marked A_1-receptor selectivity.[99]

The questions surrounding the precise mechanism of action of theophylline should give new impetus to the search for bona fide phosphodiesterase inhibitors as potential antiallergics and bronchodilators. Moreover, the existence of several forms of cyclic nucleotide phosphodiesterases having different kinetic characteristics and tissue distributions presents the intriguing possibility of developing tissue-selective (e.g., inflammatory cells, airway smooth muscle) phosphodiesterase inhibitors.[100] Indeed, the biochemical responses to one such compound, TVX 2706 (38), were evaluated in several isolated cell systems.[101] This compound was found to inhibit selectively a low K_m, cAMP-specific phosphodiesterase isozyme in rat polymorphonuclear leukocytes, perhaps accounting for its potent anti-inflammatory action in vivo.[101] The synthesis and cardiac activity of another series of selective inhibitors (39) of the low K_m, cAMP-specific phosphodiesterase, were also reported.[102]



37, R=CH_3, C_2H_5, $(CH_2)_2CH_3$



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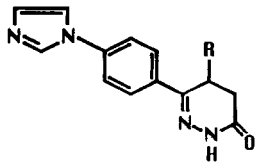
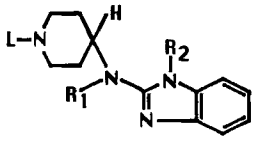


39, R=H, CH_3

An attempt to combine the activity of a β_2-adrenoceptor agonist and a phosphodiesterase inhibitor has been reported.[103]

ANTIHISTAMINES - The pharmacology and therapeutic profiles of the non-sedating histamine H_1-receptor antagonists terfenadine[104,105] and astemizole[106] were reviewed and potential advantages of these compounds over older agents were discussed.[107] The clinical efficacy of terfenadine and astemizole was compared in studies of patients with hay fever[108-111] and chronic urticaria.[112] The synthesis and pharmacology of a series of N-heterocyclic 4-piperidinamines (40), of which astemizole (41) is a member, were described.[113-115] A preliminary report appeared on the safety and efficacy of SK&F 93944 (42), a new non-sedating antihistamine, in patients with hay fever.[116] The synthesis and biologic activity of a novel class of agents combining xanthine and H_1-antihistamine moieties on the same molecule were described.[117]



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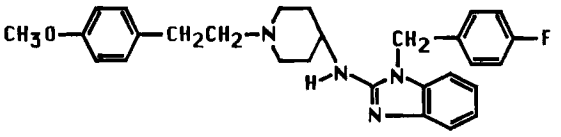

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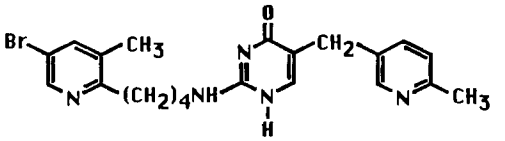

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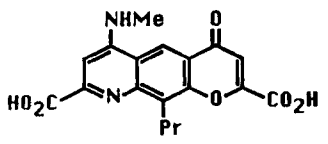
The role of histamine in producing the signs and symptoms of bronchial asthma was evaluated.[118] Historically, H_1-antihistamines have been only marginally effective in preventing antigen-induced bronchoconstriction, perhaps because the sedative effects of classical antihistamines limit their therapeutic utility, or because histamine is not a primary mediator of allergic asthma. The former proposal received support from recent studies where astemizole was shown to blunt antigen-induced bronchoconstriction in allergic asthmatics.[118,119] Furthermore, both astemizole[120] and terfenadine[121] were shown to be effective against exercise-induced asthma. Although the asthmatic response was not abolished in any of the studies cited, the results suggest that antihistamines (particularly the non-sedating agents) may have a role in the pharmacotherapy of bronchial asthma.

MEDIATOR RELEASE INHIBITORS (MRIs) - New topically and orally active MRIs continue to be reported, although the precise biochemical mechanism of action of this class of anti-allergic agents remains unknown. Nedocromil sodium (43) and minocromil (44) are topically active MRIs (rat PCA ID_{50} = 0.22 and 0.95 mg/kg intraderm., respectively). Both compounds induce phosphorylation of a 78,000 dalton mast cell protein, a process believed associated with the biochemical mechanism by which they stabilize these cells.[122] AA-673 (45), an orally active MRI, is undergoing clinical study.[123] New orally active MRIs include pyrimidinylethenyloxanilates,[124] triazoloquinoxalinediones,[125] and imidazotriazines.[126]



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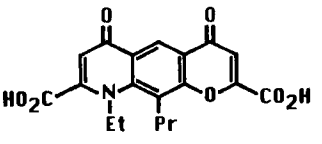

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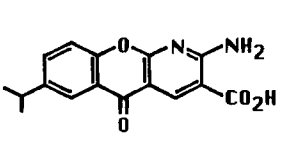

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Chapter 9. Calcium Modulators

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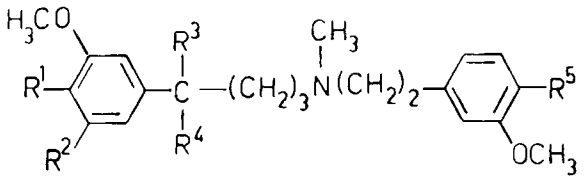
Wuppertal-Elberfeld, FRG

Introduction - Calcium channel antagonists are important cardiovascular drugs which inhibit smooth and cardiac muscle contraction by blocking the influx of calcium ions through plasma membrane channels. Comprehensive surveys of their pharmacology and current and potential therapeutic use have appeared.[1-4] The clinically well established drugs are verapamil[5], nifedipine[6] and diltiazem.[7] In addition to nicardipine, three other calcium antagonists - gallopamil[8], nitrendipine[9], nimodipine[10] - have recently become commercially available.

The discovery of 1,4-dihydropyridines (DHP) that increase the force of contraction by stimulating the influx of calcium ions in both smooth and cardiac muscle represents a major advance in the pharmacology of drugs that act directly on calcium channels.[11] Recent electrophysiological and ligand binding studies have provided new insight into the mechanism of modulation of current flow through calcium channels. The ability of dihydropyridine compounds to either promote or inhibit calcium influx and the fact that both properties are observed dose-dependently in the same dihydropyridine molecule, illustrate that these agents may really be looked at as calcium modulators.[12]

Verapamil Analogues - In addition to verapamil (1), gallopamil (2) and tiapamil (3), some new compounds such as anipamil (4), desmethoxyverapamil (5), AQ-AH 208 and UL-FS 49 have been reported.



	R^1	R^2	R^3	R^4	R^5
1	OCH_3	H	$CH(CH_3)_2$	CN	OCH_3
2	OCH_3	OCH_3	$CH(CH_3)_2$	CN	OCH_3
3	OCH_3	H	$-O_2S-CH_2-CH_2-CH_2-SO_2-$		OCH_3
4	H	H	$C_{12}H_{25}$	CN	H
5	OCH_3	H	$CH(CH_3)_2$	CN	H

Gallopamil has been marketed for the prophylaxis and long-term therapy of coronary heart disease and atrial tachyarrhythmia.[13] Fifteen mg 2 to 3 times a day significantly improved symptoms of angina pectoris.[14] Detailed voltage clamp analyses of its mechanism of calcium channel blockade have shown that, in ventricular muscle, gallopamil preferentially binds to open (activated) calcium channels.[18,19] The beneficial effect of tiapamil (RO 11-1781) in patients with coronary artery disease has been demonstrated after 200 to 800 mg of the drug.[16] Anipamil which is characterized by its long aliphatic side chain R^3 is a new verapamil derivative with a long lasting antihypertensive action.[17] From preliminary results on healthy volunteers about 100 mg p.o. anipamil, once daily, are expected to be clinically effective in an antihypertensive therapy.[18] Desmethoxyverapamil (D 888) has only been used as a pharmacological tool. The (-)-isomer is 100 times more potent than its (+)-enantiomer at inhibiting Ca^{2+}-induced tension in depolarized rat aortic strips as well as in decreasing force of contraction of cat papillary muscles.[19]

Recently, several compounds have been developed as specific bradycardic agents. One prototype is falipamil (AQ-A 39, 6) which is chemically related to verapamil. Its antianginal efficacy has recently been demonstrated in a pilot study.[20] Two congeners of falipamil are AQ-AH 208 (7) and UL-FS 49 (8). UL-FS 49 has been shown to decrease the

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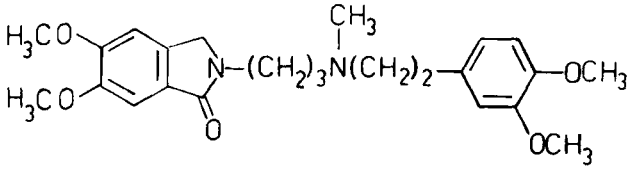

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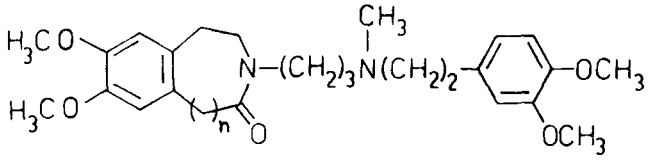
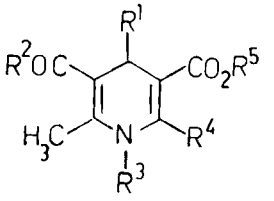


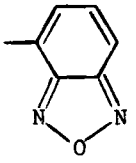
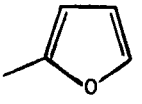
rate of spontaneously beating guinea pig atria at much lower concentrations (EC_{30} = 0.03 µg/ml) than those at which it decreased the contractility (EC_{30} = 108 µg/ml).[21] In anesthetized dogs with ischemic myocardial perfusion, both compounds produced dose-dependent reductions in heart rate without major effects on aortic blood pressure or maximal left ventricular dP/dt and improved post-stenotic myocardial perfusion particularly to the subendocardium.[22]

Nifedipine Analogues - Nitrendipine (10) has been marketed as an antihypertensive drug with a longer duration of action than nifedipine (9).[9] Single oral doses, ranging from 5 to 40 mg, produced a prompt, safe and sustained hypotensive effect in patients with mild or moderate hypertension, the maximum effect being observed 1 to 3 hours

after dosing.[23] In hypertensive patients with coronary artery disease who were inadequately controlled by a ß-blocker, a marked therapeutic response was obtained upon addition of nitrendipine.[24]

Nimodipine (11), due to its predilective effect on spasms of cerebral vessels, has become the drug of choice in patients with subarachnoid hemorrhage.[10] Its effectiveness in the prophylaxis of migraine has also been substantiated.[25] Moreover, nimodipine penetrates the blood-brain barrier and elicits some direct psychotropic activity.[26] In rodents, it has been shown to attenuate aggressive behaviour and to interfere with postural reflexes. Anxiolytic properties have been demonstrated as has been a long lasting protective effect on hypoxia-induced retrograde amnesia.[26]



	R^1	R^2	R^3	R^4	R^5
9	2-NO_2-C_6H_4	OCH_3	H	CH_3	CH_3
10	3-NO_2-C_6H_4	OCH_3	H	CH_3	C_2H_5
11	3-NO_2-C_6H_4	$OCH_2CH_2OCH_3$	H	CH_3	$CH(CH_3)_2$
12	2-NO_2-C_6H_4	$OCH_2CH(CH_3)_2$	H	CH_3	CH_3
13	3-NO_2-C_6H_4	$OCH(CH_3)_2$	H	CN	CH_3
14		OC_2H_5	H	CH_3	C_2H_5
15	"	$OCH(CH_3)_2$	H	CH_3	CH_3
16	2.3-Cl_2-C_6H_3	OC_2H_5	H	CH_3	CH_3
17	2-CF_3-C_6H_4	OC_2H_5	$(CH_2)_2$ 	CH_3	C_2H_5
18	2-NO_2-C_6H_4		H	CH_3	CH_3
19	2-Cl-C_6H_4	OCH_3	H	$CH_2O(CH_2)_2NH_2$	C_2H_5

The superior potency of calcium antagonistic 1,4-dihydropyridines has initiated the development of a large number of related analogues, primarily as antianginal and/or antihypertensive agents. Nisoldipine (BAY K 5552, 12) is one of the most potent blockers of voltage-dependent Ca^{2+}-channels (IC_{50}=1.1x10^{-9} M) and is characterized by its predominant vasodilating effects on coronary and peripheral blood vessels.[27-28] KCl-induced contractions of aortic strips treated with nisoldipine (2.9x10^{-7} M) remained inhibited for 15 hours.[27] Nilvadipine (FR-34235, 13) demonstrates a similar effect as nifedipine on KCl-induced responses in venous smooth muscle. However, it is much more potent than nifedipine against contractions which are mediated by activation of postsynaptic α_1-adrenoceptors.[29] Darodipine (PY 108-068, 14) appears to change the rank order of the negative chronotropic and negative inotropic effects of calcium antagonists.[30] Isradipine (PN 200-110, 15) has a longer duration of action than darodipine and has been shown to cross the blood brain barrier without stimulating any CNS effects.[31] The earlier report on felodipine (H 154-82, 16) suggesting interaction with calmodulin[32] as the basis of its vasodilating activity has been challenged, mainly due to the different concentration range of its calcium antagonism and calmodulin binding.[33,34] *In vitro* studies of flordipine (RH 2906, 17) have provided some evidence that it is metabolized to (not yet identified) compounds which may contribute to its *in vivo* antihypertensive activity.[35] MDL-72567 (18), a "keto-dihydropyridine", has been suggested to induce, in contrast to nifedipine, in vivo a depressant effect on the sinus node.[36]

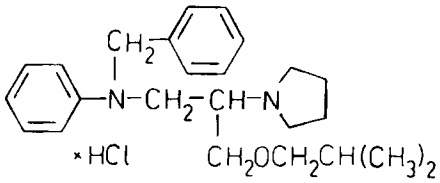
Amlodipine (UK-48340, 19) is a new antihypertensive and antianginal DHP derivative on a once-daily dose basis. In hypertensive dogs 0.5 to 2 mg/kg amlodipine reduce the blood pressure gradually, with maximum reduction occurring between 3.5 to 7 hours after application.[37] Bioavailability in dogs has been demonstrated to approach 100%.[38]

Other Structures - Bepridil (20) has recently been confirmed to inhibit the potential-operated calcium channel and related isometric tension development in vascular smooth muscle.[39,40] In isolated cardiac preparations as well as in *in vivo* studies the drug has been shown to be a selective coronary vasodilator.[41,42] At concentrations which antagonize calcium dependent mechanisms in vascular smooth muscle it appears to inhibit the activity of calmodulin.[43] Clinical experience has proven that a daily dose of 300 to 400 mg bepridil is effective in the management of stable angina.[44]

There is some evidence that in addition to blocking slow channels bepridil also influences fast sodium currents.[45] This dual action in myocardial cells implies that it is a class I as well as a class IV antiarrhythmic agent according to the Vaughan-Williams classification.[46] In anesthetized dogs bepridil increases the refractoriness of atrial myocardium, atrial ventricular nodal tissue and ventricular myocardium.[47-49] It prevents and/or terminates experimental supraven-

tricular[45] and ventricular[50,51] arrhythmias in a variety of animal models and increases the ventricular fibrillation threshold during both unobstructed coronary blood flow and regional myocardial ischemia.[50]

HA-1004 (21) appears to be a novel calcium antagonist with a selective vasodilatory action. In depolarized rabbit aorta it produced a competitive inhibition of calcium induced contraction.[52] Isolated guinea pig left atria or right ventricular papillary muscles did not respond to the drug. Due to its antagonizing effect on phenylephrine induced contractions of rabbit aorta in the absence of extracellular calcium, it has been suggested that HA-1004 affects intracellular rather than extracellular calcium. The in vitro inhibition of agonist induced contractions of canine renal arteries has been confirmed by an increase of renal blood flow in anesthetized dogs.[53]



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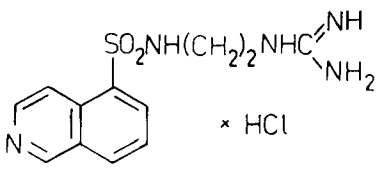

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Mechanistic Aspects - It is well established that calcium channel antagonists serve as potent and selective inhibitors of plasmalemmal Ca^{2+}-entry through voltage-depedent calcium channels.[54] Evidence for a specific mode of action is based on structure activity relationships, including stereoselectivity, that have been described for the major classes of these agents.[55] The existence of discrete binding sites for calcium antagonists and agonists has been established by intensive studies of radio-labeled, mainly 1,4-dihydropyridine-type, ligand binding to membrane preparations as well as to intact cells.[56-59] Thus, specific, high affinity, reversible and stereoselective binding is found in a variety of tissues including vascular and non-vascular smooth muscle, cardiac muscle, skeletal muscle and brain.

It has been demonstrated on the basis of X-ray crystallographic analyses of 1,4-dihydropyridines with restricted conformational flexibility that the DHP molecule interacts with the binding site preferably in a conformation in which the plane of the phenyl ring bisects the DHP ring.[60]

Correlations between tissue binding and pharmacological activities have been sought in a number of systems for which [^{3}H]-1,4-dihydropyridine binding has been demonstrated. The best correlations are those for smooth muscle where essentially 1:1 correlations can be obtained between inhibition of binding and inhibition of K^+-depolari-

zation-induced mechanical response.[61-63] In myocardium, however, the equilibrium dissociation constant K_D of [^{3}H] -nitrendipine binding is 2 to 3 orders of magnitude lower than the concentration of nitrendipine which produces half maximal pharmacological effects.[64] Electrophysiological data suggest that calcium channel blockade by nisoldipine, nitrendipine and nicardipine in heart muscle is strongly modulated by membrane potential, with blockade being more pronounced at less negative holding potentials.[65,66] This voltage dependence has been interpreted in terms of the modulated receptor hypothesis and suggests that the high affinity site probably represents binding to and stabilizing of the inactivated state of the channel in which no openings occur. Recent studies of changes in binding to intact polarized and depolarized ventricular myocytes have demonstrated that with depolarization the number of [^{3}H] -nitrendipine binding sites (B_{max}) is significantly increased.[67] However, there was no difference in K_D between polarized and depolarized cells.

Competition of [^{3}H] -1,4-dihydropyridine binding with DHPs is consistent with competitive antagonism. This is also true for the competitive interactions between 1,4-dihydropyridine agonists and antagonists indicating that they bind to the same calcium channel receptor site.[58,59,68] The inhibition of [^{3}H] -1,4-dihydropyridine binding by other calcium antagonists has disclosed apparent different sites of action of these drugs. It is now generally agreed that verapamil, gallopamil and desmethoxyverapamil act at a site distinct from the 1,4-dihydropyridine binding site and that they should be classified as negative heterotropic allosteric effectors due to their inhibition of [^{3}H] -1,4-dihydropyridine binding.[63] In marked contrast, diltiazem actually stimulates 1,4-dihydropyridine binding over its pharmacologically effective dose range either by increasing B_{max} (smooth muscle, skeletal muscle) or decreasing K_D (cardiac tissue, brain).[61,69] This positive heterotropic allosteric interaction between diltiazem and the 1,4-dihydropyridines could also be demonstrated in tissue experiments, both in cardiac and smooth muscle.[70,71]

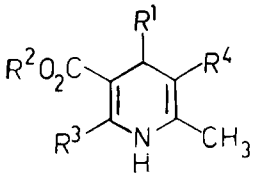
[^{3}H] -1,4-dihydropyridines have been used to determine the molecular properties of the binding site in the Ca^{2+}-channel.[72-74] In skeletal muscle, radiation-inactivation experiments have shown that the size of the dihydropyridine binding domain is decreased in the presence of diltiazem.[75] It has been suggested that the channel may be composed of subunits and that channel drugs not only alter channel function but simultaneously change the architecture of the ionic pore. The model of three drug receptor sites which are linked to each other (and to calcium binding sites) via heterotropic allosteric coupling mechanisms has recently been described.[76]

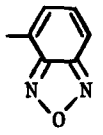
Calcium Agonists - Only minor changes of the 1,4-dihydropyridine structure led to compounds which exhibit pharmacological actions opposite to those of nifedipine: instead of inhibiting they increase the force of contraction.[77,78] BAY K 8644 (22) is the most thoroughly

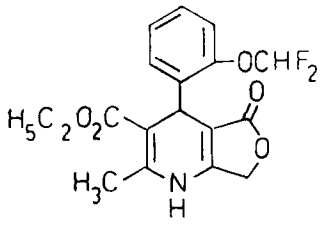
studied prototype of these agents. It exerts both *in vitro* and *in vivo* positive inotropic, vasoconstrictive and moderate positive chronotropic activity in a concentration range from 10^{-9} to 10^{-7} M.[11,79,80] Using $^{45}Ca^{2+}$, increased Ca-influx during cellular depolarization could be correlated with enhanced electromechanical coupling.[81,82]

Electrophysiological studies have shown that the prolongation of the plateau phase of the cardiac action potential is indicative of increased Ca^{2+}-influx and the consequent increase in contractility.[83] Investigations of the single Ca^{2+}-channel kinetics with BAY K 8644 have revealed that this compound dose-dependently keeps the channel in the open state for a longer period.[84-86]

In addition to BAY K 8644 other dihydropyridines with calcium-agonistic properties have been disclosed. CGP-28392 (26) also exerts positive inotropic and vasoconstricting effects.[87] The active dose-range is about one order of magnitude higher than that of BAY K 8644. *In vivo* its positive inotropic response appears to be less pronounced than its vasoconstrictive properties.[88] The main pharmacological effect of YC-170 (23) is vascular contraction. It is about 3 times less potent than CGP-28392 and displays minimal positive inotropic and no positive chronotropic activity.[89,90] H 160/51 (24) has also been shown to be a calcium agonistic dihydropyridine with an ED_{50} of 1 µM in cat papillary muscles and in a portal vein preparation.[91,92] Compound 202-791 (25) exerts positive inotropic and vasoconstricting effects by increasing Ca^{2+}-influx.[93] As with BAY K 8644 it has been shown that the Ca^{2+}-modulating effect of 202-791 is strongly dependent on membrane potential. It is an agonist under well-polarized conditions. However,



	R^1	R^2	R^3	R^4
22	2-CF_3-C_6H_4	CH_3	CH_3	NO_2
23	2-Cl-C_6H_4	$(CH_2)_2$- 	CH_3	$CONH-C_6H_5$
24	2-Cl-C_6H_4	C_2H_5	NH_2	H
25		$CH(CH_3)_2$	CH_3	NO_2



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when the cell is depolarized, it is converted to an antagonist.[93,94] This result, together with the finding that high drug concentrations also reverse the calcium agonism to calcium antagonism, brought up hypotheses on the molecular basis of the calcium modulating properties of 1,4-dihydropyridines.[12,95]

The recent observation that only the (-)-enantiomer of BAY K 8644 and the (+)-enantiomer of 202-791 are responsible for the calcium agonistic effect while the optical antipodes are calcium antagonists, strongly underscore the close structural vicinity of calcium antagonism and calcium agonism in this class of drugs.[93,96]

Conclusions - Several calcium antagonists are under development. In addition to pure vascular effects parenchymal activity, e.g. in the brain, could be demonstrated. Knowledge of the basic mechanism of modulation of calcium currents has considerably increased due to electrophysiological and ligand binding studies as well as to the discovery of calcium agonists. The latter are pharmacological tools and provide a novel mechanism of positive inotropic activity which may lead to new drugs for the treatment of congestive heart failure.

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Chapter 10. Class I And III Antiarrhythmic Drugs

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Introduction - Nearly 10 years have elapsed since the local anesthetic antiarrhythmic drugs (class I) were reviewed in this series.[1] Drugs that prolong action potential duration (APD) selectively (class III) were last discussed in 1983.[2] An abundance of reviews on the electrophysiological basis of cardiac arrhythmias[3-5] and therapeutic use of antiarrhythmic drugs[6-8] have appeared recently. The large volume of new information has mandated that we limit our discussion to class I and III agents, with emphasis on new compounds that appeared in the literature during the last three years. However, recent reports relevant to the mechanism of action of older agents will also be mentioned.

The Vaughan Williams classification recognizes four distinct antiarrhythmic groups.[9] Class I agents may be further divided into three subgroups on the basis of effects on APD.[10] The potent conduction depressants flecainide, encainide and lorcainide are Ic agents that have little effect on APD, while lidocaine, tocainide and mexiletine are examples of Ib compounds that shorten APD. Quinidine, disopyramide and procainamide represent Ia agents that prolong repolarization.[11] In addition, the observation that local anesthetics possess unique affinities for the sodium channel depending on whether the channel is in the resting, open or inactivated state (modulated-receptor hypothesis),[12] has provided added biophysical support for the subclassification of class I drugs. Thus, Ib agents tend to rapidly associate with and dissociate from open and/or inactivated sodium channels with time constants in the 100-300 msec range, while Ic agents require tens of seconds for block onset and recovery; Ia agents are intermediate.[11,13]

The emergence of programmed electrical stimulation (PES) techniques for the clinical evaluation of antiarrhythmic drugs continues to have an important impact on the way new drugs are tested.[14] A positive response to a drug in a standardized PES protocol portends a high probability of success upon long-term treatment, although a negative drug response does not necessarily predict failure.[15,16] In patients with sporadic, life-threatening arrhythmias, PES rather than Holter recording may be the preferred method of drug evaluation.[17] Improved animal models are available to simulate PES-induced arrhythmias in humans.[18,19] The induced tachycardias seem to be reentrant in origin and respond predictably to antiarrhythmic drugs.[20] Nevertheless, some evidence suggests that, in terms of rate, morphology and reproducibility, these models may not adequately mimic the typical monomorphic reentrant ventricular tachycardia that appears to be the best clinical predictor of drug efficacy.[21,22] An animal model that causes "sudden cardiac death" may also be relevant to new drug evaluation.[23] This model involves electrically induced thrombus generation within the circumflex coronary artery in dogs having had a prior infarct in the distribution of the left anterior descending coronary artery.

Class I: Update on established agents - Recent reviews on the pharmacology and mechanism of action of standard agents such as quinidine,[24] procainamide,[24,25] disopyramide[24] and lidocaine[26] have appeared. Symposia and reviews of the electrophysiology and therapeutic use of newer type I agents including propafenone,[27] pirmenol,[28] tocainide,[29] mexilitine,[30] lorcainide,[31] encainide[32] and flecainide[33] have also been published.

Class I agents interact weakly with resting sodium channels at normal resting membrane potentials; activation is required before these agents block (use-dependency).[12,13] Lidocaine interacts with both open and inactivated channels, whereas quinidine and amiodarone preferentially interact with open and inactive channels, respectively.[12] Separate mechanisms mediating resting and use-dependent block were distinguished using propafenone and structural analogs.[34] Regardless of which channel state is preferentially blocked by class I drugs, the unblocking rate directly determines the amount of depression present at normal heart rates. Since the Ic agents like flecainide,[13,35] encainide,[13] and lorcainide[35] possess long recovery time constants, these compounds tend to depress conduction velocity in normal and especially depolarized myocardium at all physiologically relevant heart rates. The Ib agents with short time constants show little accumulation of block between impulses; early or rapid impulses are preferentially blocked.[11,12] In general, small molecular size, high lipid solubility, and low pKa favor a fast unblocking rate for class I drugs.[13,36,37]

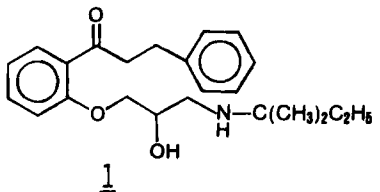
Competition studies have added strong support to the notion that class I drugs act on a common receptor site, but possess selective affinities for different sodium channel states. For example, high concentrations of lidocaine relieved sodium channel block caused by bupivicaine, an agent that acts on inactivated channels and dissociates much more slowly than lidocaine.[38] Competition interactions between propafenone and lidocaine acting on different sodium channel states have also been described.[39] The use-dependent effects of lidocaine in dog Purkinje fibers were specifically inhibited by SC 7214, a monoclonal antibody against the electroplax fast sodium channel. However, in this case, SC 7214 and lidocaine did not appear to interact at a common site within the channel.[40]

Metabolites of several antiarrhythmic drugs are pharmacologically important and continue to be extensively investigated. Encainide's two major metabolites, 3-methoxy-O-demethyl encainide and O-demethyl encainide (ODE) possess antiarrhythmic[41] and class I electrophysiological properties.[42] ODE, especially in ischemic tissues, may be likely to cause profound conduction depression[43] and, like flecainide,[44] may induce arrhythmias in dogs with infarcted hearts.[41] In anesthetized dogs, the electrophysiological effects of lorcainide and its N-dealkylated metabolite, norlorcainide, are qualitatively and quantitatively similar.[45] However, the O-dealkylated metabolites of flecainide are only 1/2 to 1/10 as active as flecainide.[46] Acecainide (NAPA), the rapidly formed N-acetyl metabolite of the class I agent, procainamide, is a selective class III drug.[47] Since NAPA, unlike procainamide, does not induce a lupus-like syndrome, it has been investigated clinically, but the results have been equivocal.[48,49] The pharmacokinetics and electrophysiology of the desisopropyl metabolite of disopyramide have been investigated.[50] The authors concluded that this metabolite contributes significantly to the anticholinergic side-effects seen during disopyramide therapy.

Effects of stereochemistry on activity of chiral antiarrhythmics remains an underdeveloped area. The l-isomer of disopyramide was found to be more potent than its enantiomer in slowing sinus rate and increasing AV-nodal refractoriness.[51] The two enantiomers of flecainide were found to be equipotent in suppressing chloroform-induced ventricular fibrillation (VF) in mice and ouabain-induced ventricular tachycardia (VT) in dogs.[52] Conformational perturbations produced by an intramolecular hydrogen bond between the amide and pyridine moieties of disopyramide appear to increase the ratio of antiarrhythmic to anticholinergic activity; model compounds were prepared to test this hypothesis.[53]

Antiarrhythmic effects of quaternary ammonium class I agents were further characterized in vivo. The quaternary analogs of propranolol, UM-272[54] or UM-424,[55] were studied after oral administration to dogs with chronic infarcts. UM-272 reduced the incidence of VF in the acute thrombosis model but not the accompanying VT. UM-272 possessed typical Ia electrophysiological effects including marked use-dependent sodium channel block with a block recovery half-time of 7 sec.[56] UM-424 abolished VT induced by PES by decreasing conduction preferentially in the ischemic zone and increasing refractoriness in normal zones.[55]

Class I: Recently reported new chemical entities - Several new class I agents possessing the β-antagonist pharmacophore have been reported. Diprafenone (1) is a recently reported propafenone congener. It is more potent than propafenone, and displays maximum antiarrhythmic activity at doses which have only minor depressant effects on intraventricular conduction and hemodynamics.[57] Compound 711389-S (2) was active against aconitine and ouabain-induced arrhythmias.[58,59] Detailed electrophysiological evaluation in guinea pig muscle indicated 2 is a class Ia antiarrhythmic with less anticholinergic activity than either disopyramide or quinidine.[60] Several lines of evidence indicate β-receptors are not involved in its antiarrhythmic effect. In guinea pig atria 2 was considerably less potent than propranolol as a β-antagonist (pA_2's = 5.29 and 8.62, respectively), but was more potent as an antiarrhythmic; moreover, there were no significant differences in



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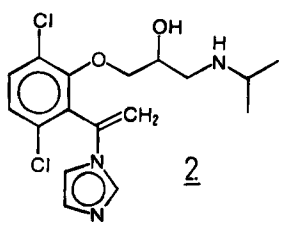

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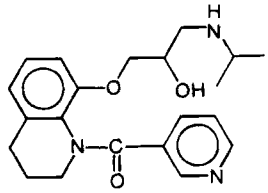

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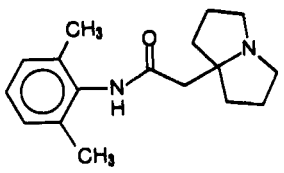
antiarrhythmic activities of the R and S enantiomers.[58] Nicainoprol (RU-42924, 3) is a class Ib drug with moderate β-blocking activity; unlike propafenone, 3 has no significant negative inotropic potential.[61,62]

A number of compounds bearing the 2,6-dimethylanilide substructure of lidocaine have been reported. SUN-1165 (4) contains the highly basic pyrrolizidinyl moiety instead of the less basic diethylamine group of lidocaine.[63] Electrophysiological studies in vitro revealed that 4 is a Class Ib drug.[64,65] Compared to lidocaine and disopyramide, the compound had less CNS toxicity and anticholinergic activity, respectively.[66] Another Ib lidocaine-like agent is recainam (WY-423262) (5). Therapeutic i.v. doses failed to produce CNS or negative inotropic side-effects in

dogs,[67] and the drug safely and effectively reduced PVC's in man.[68] Droxicainide (ALS-1249, 6) was reported to have anti-arrhythmic and local anesthetic properties that are qualitatively similar to lidocaine,[69,70] but with a better therapeutic index in 24 hr infarct dogs than lidocaine.[71] The piperidine and azepine analogs of droxicanide which lack the hydroxyethyl moiety are also active.[72]



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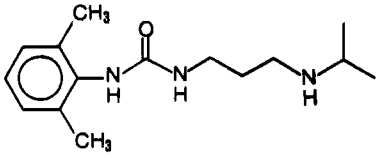

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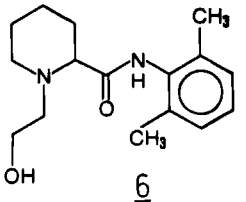

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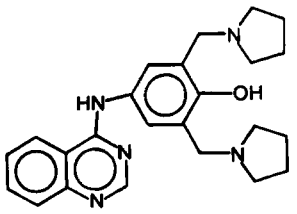
Stout and co-workers have conducted extensive studies on the SAR of changrolin (7) to eliminate untoward side-effects (skin discoloration and anticholinergic activity) of the parent compound. The bis(pyrrolidinylmethyl)phenol portion of 7 was optimal for antiarrhythmic activity, but the quinazoline moiety could be varied considerably while maintaining activity.[73] Replacement of the quinazoline ring with a variety of substituted benzoyl groups resulted in several potent antiarrhythmics with minimal anticholinergic activity.[74] Further SAR studies led to the selection of ACC-9358 (8) for development.[75] Electrophysiologically, these compounds appear to be Class I agents.[76,77]



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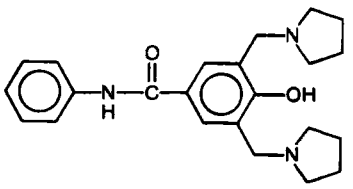

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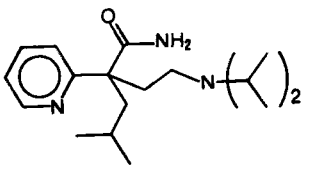

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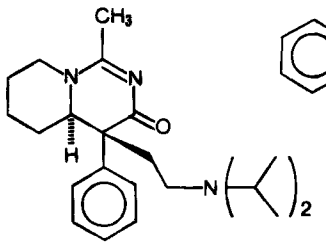
Disopyramide continues to serve as a prototype for new antiarrhythmics. A series of compounds in which the phenyl group of disopyramide was replaced with alkyl moieties was synthesized.[78] One of the compounds in this series, propisomide (CM-7857, 9), was reported to possess a longer duration of action, fewer neurologic and gastrointestinal side-effects and less anticholinergic activity than disopyramide.[79] As with disopyramide, the major metabolite in both plasma and urine was the mono-N-dealkylated analog.[80] A single oral dose of 9 was compared to a variety of other antiarrhythmics in 10 patients. The compound was effective in 6; it increased PQ intervals, and had no effects on QRS or QTc intervals.[81] The pyridyl portion of disopyramide can be modified considerably, and some reduced bicyclic analogs were prepared.[82] The optimal compound of the series, SC-36602 (10), is currently in clinical trials. A series of animal studies revealed that 10 is a Class I agent; moreover, it did not produce adverse CNS or negative inotropic effects, and possessed only about 7% of the anticholinergic activity of disopyramide.[83-85]

AHR-10718 (11) is the optimal compound in a series of aminoethylureas that suppressed arrhythmias in the ouabain-intoxicated and Harris dog models. The drug caused a use-dependent decrease in action potential upstroke velocity (V_{max}), Purkinje fiber conduction velocity and APD.[86] Carocainide (MD770207, 12), a benzofuran antiarrhythmic, decreased V_{max} in isolated papillary muscles and Purkinje fibers; in Purkinje fibers it decreased the plateau amplitude and APD.[87] The compound antagonized digitalis- and infarction-induced arrhythmias in dogs.[88-90] A report on human metabolism and disposition has appeared.[91]



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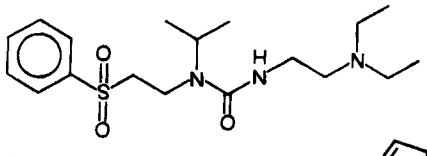

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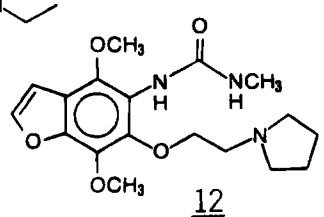
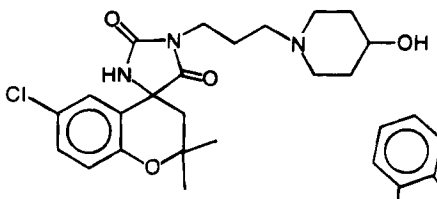


12

E-0747 (13) was more potent than quinidine, disopyramide, lidocaine, or phenytoin. Moreover, at therapeutic doses, the compound appeared to have low potential for cardiodepression.[92] Indecainide (LY135837, 14), is a potent class Ic antiarrhythmic agent[93,94] with an exceptionally long half-time (52 sec) for recovery from Na^+ channel block.[93] This compound was more potent than either aprindine or disopyramide against ouabain or Harris arrhythmias in dogs.[95] Reports on its metabolism have appeared.[96] Quinacainol (PK-10139, 15) was 5 to 10-fold more potent, and longer-acting than quinidine in various animal models.[97] In contrast to quinidine, there was no significant interaction with digoxin.[98] In dogs 15 was 40-50 times more potent than quinidine in decreasing conduction velocity throughout the myocardium.[99]



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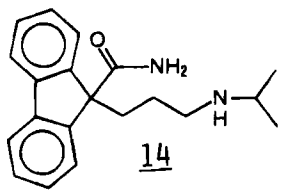

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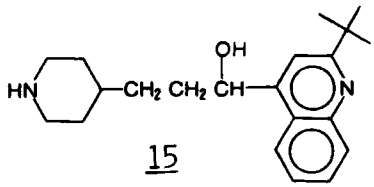

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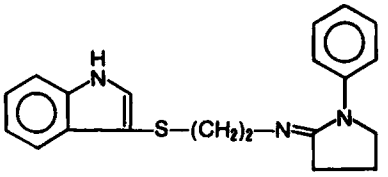
Antifibrillatory activity of the class I antiarrhythmic McN-4130 (16) has been investigated. It protected against VF in ischemic porcine hearts, increased ventricular fibrillation threshold (VFT) in dogs, and was highly effective in terminating acetylcholine-induced atrial fibrillation.[100] In canine Purkinje fibers 16 caused a rate-dependent decrease in V_{max} and prolonged APD_{100}.[101] Stirocainide (EGYT-1855, 17) also decreased V_{max} in a use-dependent fashion and shortened the plateau of Purkinje fiber action potential without influencing either the resting potential or the amplitude of the action potential.[102] Recent animal studies indicated the drug was highly effective in suppressing arrhythmias following acute infarction.[103]



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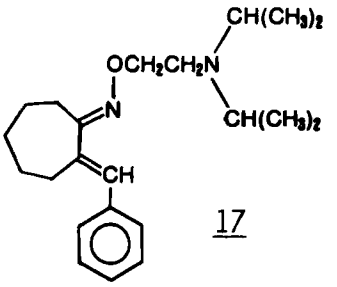

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Class III: Update on established agents - By slowing conduction and inhibiting automaticity, class I drugs slow VT rate and control the trigger mechanisms for initiating VT. In contrast, class III agents act by homogeneously increasing refractoriness without slowing conduction, thus interrupting reentrant pathways and decreasing the vulnerability to VT/VF.[104-106] The distinction between the antiectopic effects of class I drugs and the antifibrillatory effects of class III drugs, especially bretylium, has also been emphasized.[107,108] Summaries of the pharmacology and clinical use of the only marketed class III agents, amiodarone[109,110] and bretylium,[111] have appeared.

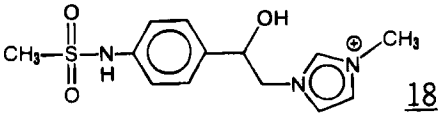
Clofilium[112,113] and d-sotalol[114,115] have selective class III properties in patients and suppress PES-induced VT with minimal side effects. Bretylium[116,117] and meobentine[118] were only marginally successful in patients with PES-induced sustained VT. The incidence of GI side effects and hypotension was especially troublesome with meobentine. Bretylium (30 mg/kg, infused over 24 hr) prevented the decrease in effective refractory period (ERP) accompanying acute coronary artery occlusion, thereby reducing refractory period dispersion upon reperfusion and preventing reperfusion arrhythmias.[119] Large doses of bretylium (10 mg/kg every 12 hr for 2 days) abolished PES-induced VT in dogs with chronic infarcts and reduced the occurrence of sudden death upon acute thrombosis.[120] Acute i.v. administration of bretylium to conscious dogs (10 mg/kg) did not prevent reperfusion arrhythmias[119] and failed to elevate VFT in anesthetized dogs (10-30 mg/kg).[121] A positive effect of acute bretylium (10 mg/kg, i.v.) on reperfusion arrhythmias in dogs was detected only if the area of myocardial ischemia was considered.[122] The mechanism of action of bretylium remains elusive. A reduction in dispersion of refractory period[123] and conduction velocity[124] between ischemic and normal tissues has been reported. Clofilium, a selective class III agent without antisympathetic effects,[125] increased refractoriness and VFT in both dogs[106,126] and cats[123] but was less effective than bretylium in preventing VF during acute ischemia.[123,126] Thus, the antiadrenergic effect of bretylium[127] appears to be required for optimal antiarrhythmic effects in acute ischemia models.[128] At pharmacologically relevant concentrations, bretylium also exerted antimuscarinic effects in guinea pig atria and bound to muscarinic receptors in rat heart.[129]

In isolated cardiac tissue, d,l-sotalol (10^{-6}M - 10^{-4}M) increased APD[130] and refractoriness, even in the presence of elevated K^+.[131] The effects of d,l-sotalol were unrelated to β-blockade and were mediated by decreases in background and plateau potassium current.[130] Although the class III actions of d,l-sotalol could not be correlated with its antiarrhythmic effects in ischemic perfused guinea-pig hearts,[132] class III effects clearly mediated its antiarrhythmic and antifibrillatory

effects in chronically ischemic dogs.[133,134] Both isomers of sotalol increased APD in vitro.[130] d-Sotalol, 2-8 mg/kg administered acutely[135] or 8 mg/kg administered every 6 hr for 24 hr,[136] suppressed the induction of VT in infarcted dogs, increased refractoriness and prevented development of VF in response to acute thrombosis. The β-blocking isomer, l-sotalol, had similar effects except that the PR interval was lengthened.[136]

Amiodarone is a fairly selective class III agent when administered chronically. However, superfusion of guinea pig ventricular muscle with high concentrations of amiodarone (88 μM) caused rate-dependent reductions in V_{max};[137] lower concentrations (15 μM) depressed conduction through the rabbit AV node and interfered with sinus node function by a non-class III mechanism.[138] Short-term i.v. infusion of amiodarone (10 mg/kg/hr) in dogs with chronic infarcts was less effective than long term oral therapy (10 mg/kg/day for 24 days) in preventing VF due to acute thrombosis.[139] The enhanced efficacy of chronic therapy was accompanied by greater increases in the QTc interval, although refractoriness was not measured. Amiodarone is also a fairly potent non-competitive muscarinic antagonist in heart tissue (K_i=4-6 μM).[140] Thus, the mechanism of action of amiodarone is complex and probably involves a combination of metabolic, autonomic and direct effects. In rats, meobentine prolonged APD in vitro, increased refractoriness and reduced arrhythmias associated with coronary artery occlusion in vivo .[141] In dogs with chronic infarcts, meobentine increased VFT but did not protect conscious dogs from developing VT/VF in response to PES or acute thrombus formation.[142]

Class III: Recently reported new chemical entities. - A series of phenethylimidazolium analogs of sotolol were prepared. Compound CK-1649C (18) prolonged APD of isolated canine Purkinje fibers and functional refractory period of isolated ventricular muscle fibers; there was no effect on cardiac conduction, and electrophysiologically, the two stereoisomers were indistinguishable.[143] Compound 18 was orally bioavailable and antagonized PES-induced arrhythmias in dogs. The SAR leading to the discovery of a series of clofilium-like class III antiarrhythmics was described. Removal of an N-ethyl group from clofilium (19) a quaternary ammonium derivative, results in the formation of LY97119 (20), an agent devoid of class III antiarrhythmic activity. However, LY97241 (21), the nitro congener of 20, is even more potent as a class III agent than clofilium.[106]



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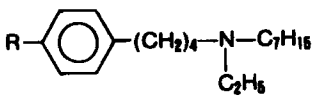

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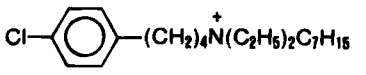

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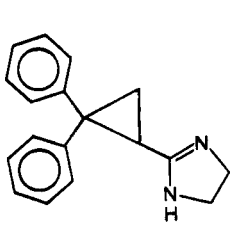
Agents with Mixed Activity - Propafenone has about 1/40 to 1/50 the potency of propranolol as a β-blocker;[144] however, because of the large doses administered, this activity probably contributes to its clinical antiarrhythmic efficacy.[144,145] Cibenzoline (22) decreased automaticity arising from depolarized membrane potentials[146] and prolonged AH interval and APD in muscle of isolated rabbit tissue,[147] suggesting the involvement of class III and IV activity. The compound is a potent sodium channel blocker in cardiac tissues in vitro [146] and has no effect

on AH interval in dogs *in vivo*,[148] suggesting that additional effects of 22 are probably of minor importance. Compound 22 is being evaluated extensively in clinical trials, and a compendium of results obtained to-date has appeared.[149] Bepridil had little effect on normal canine Purkinje fiber APD or V_{max}, but decreased V_{max} of ouabain-intoxicated fibers.[150] The antiarrhythmic effects of bepridil *in vivo* were accompanied by increased intramyocardial conduction time, QRS duration, QTc interval and refractoriness in dogs with chronic infarcts.[151] Thus, class I, III and IV activities probably contribute to the antiarrhythmic effects of this agent. The neuroleptic agent, melperone, possessed class III activity in isolated rabbit atrial and ventricular tissues but also inhibited V_{max}, especially in Purkinje fibers and the ventricle.[147]

Asocainol (GOE-4704A, 23), has recently been advanced to clinical trials.[152] This agent appeared to be a mixed class I/IV antiarrhythmic in isolated guinea pig papillary muscles. Its block of sodium and calcium channels was not stereospecific.[153] Another example of a dual class I/IV agent is BRL-31660 (24).[154] The combination of class I and IV mechanisms may lead to enhanced antiarrhythmic efficacy, but marked negative inotropic activity is a potential liability. A series of amino substituted steroids was investigated and CCI-22277 was found to be more active than lidocaine, disopyramide or aminosteroid Org 6001 in the rat aconitine test.[155] Compound CCI-22277 was reported to be a class I antiarrhythmic with an long recovery time constant; however, additional class IV action at high concentrations was evident.[156]



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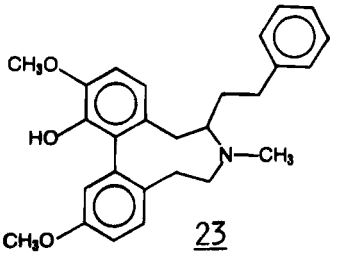

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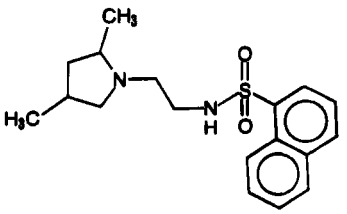

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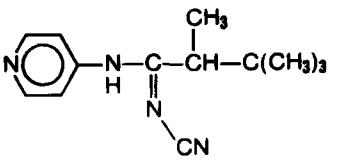
Miscellaneous Agents - A variety of vasodilator substances have activity against arrhythmias during acute coronary artery occlusion in dogs.[157] Nicorandil (SG-75, 25) shortens APD and reduces automaticity, effects mediated by an increase in K^+ conductance.[158] The antihypertensive agent pinacidil (26) markedly decreased ventricular ectopic beats in conscious dogs 24 hr following coronary artery occlusion;[159] other vasodilators, such as hydralazine or nitroprusside were inactive. Trapidil (27), an antianginal drug, significantly increased the amount of aconitine or ouabain needed for induction of ventricular arrhythmias in rats and guinea pigs. In isolated rabbit ventricular muscle cells, trapidil shortened APD and prolonged ERP.[160] Comprehensive reviews on the basic electrophysiology[161] and therapeutic uses[162] of adenosine in supraventricular arrhythmias have appeared. This nucleoside has also demonstrated efficacy against ventricular arrhythmias in rats.[163] A prostacyclin "potentiator", nafazatrom (BAY-S6575), reduced infarct size and markedly decreased arrhythmias following acute coronary artery occlusion in rats.[164] The thromboxane synthetase inhibitor, dazoxiben decreased reperfusion VF but not occlusion arrhythmias in anesthetized dogs.[165] Moreover, additional thromboxane synthetase inhibitors, including furagrelate (U-63557A, 28), markedly decreased VF upon acute coronary artery occlusion in conscious dogs. The antifibrillatory effect was blocked by indomethacin, suggesting the involvement of prostacyclin.[166]



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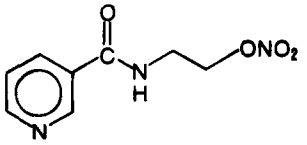

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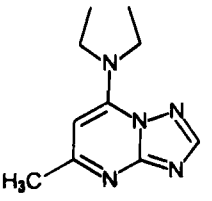

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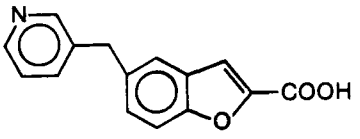

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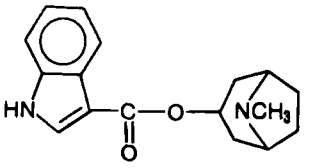

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Phencyclidine[167] and amiloride[168] produced selective class III effects in isolated Purkinje fibers. Phencyclidine's effect is likely related to block of K^+ currents by the uncharged amine.[167] In studies investigating the role of endogenous opioids in acute ischemia, naloxone failed to protect pigs against arrhythmias associated with acute coronary artery occlusion and release.[169] The potent serotonin antagonist ICS205-930 (29) was found to have a direct, class I electrophysiological effect on the heart, and protected rats from reperfusion-induced VF and VT.[170] The contribution, if any, of its serotonin antagonist properties to this cardioprotective effect has not been delineated.

Proarrhythmia - The fact that antiarrhythmic drugs can induce arrhythmias under some circumstances is now well-appreciated.[171,172] In general, the phenomenon can occur with any antiarrhythmic drug of any class. Many reports have focused on investigational class I agents, possibly because they are scrutinized carefully and used more often in patients that are debilitated and refractory to standard drugs.[173] However, an excessively prolonged QTc interval may indicate enhanced disparity of recovery times and may also be arrhythmogenic.[174] The moderate increase in QTc that accompanies the antiarrhythmic effect of most class III drugs probably signifies a homogeneous increase in ventricular repolarization.[175] Nevertheless, development of class III drugs that increase refractoriness with minimal increases in QTc would seem worthwhile. At present it is difficult or impossible to predict when and if a given patient will experience a proarrhythmic response with either class I or III drugs. Because aggravation of arrhythmias is of paramount importance in assessing the therapeutic potential of antiarrhythmic agents, preclinical methodology to determine the proarrhythmic liability of new drugs will be mandatory. A few investigators have already attempted to address this issue.[176,177]

Future Directions - Complex and/or frequent arrhythmias in the post-myocardial infarction population serves as an independent marker for increased 1 and 2 year mortality.[178] However, no studies have yet shown that prophylactic therapy with class I or III agents can decrease mortality in high-risk groups.[110,179] Carefully controlled large-scale clinical trials now being planned should address the value of arrhythmia suppression in reducing mortality in patients with potentially lethal arrhythmias. Meanwhile, the safe reduction of life-threatening arrhythmias in high-risk patients should remain an important goal for drug development efforts. In addition, nonpharmacologic treatments such as automatic implantable cardioverter-defibrillators have been developed.[180] Although these non-drug options may seem to compete with drug therapy, new drugs might actually complement such devices. Drugs that reduce the frequency of device operation or reduce current delivery could have an important impact on the design of future electrical devices. For example, although lidocaine[181] and encainide[182] have been shown to increase the internal defibrillation threshold, some class III agents such as d-sotalol and clofilium[183] have the opposite effect. Finally, the availability of implantable devices may facilitate drug development efforts by alleviating ethical concerns which currently limit placebo-controlled studies of new antiarrhythmic drugs in patients with serious arrhythmias.

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Chapter 11. Pharmacological Approaches in Acute Stroke

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INTRODUCTION

Stroke is the third leading cause of death in the United States today,[1] and yet there remains no generally agreed upon effective treatment for this devastating neurological disorder. It has been estimated that each year 400,000 Americans suffer a stroke and two-thirds of the survivors are permanently disabled.[2] Acute and long-term disability from stroke costs the nation over $8 billion each year.[3]

Stroke is caused by an abrupt interruption in blood supply to part of the brain, resulting in ischemia and cerebral tissue necrosis. Most often the interruption in cerebral blood flow is caused by an atherosclerotic arterial narrowing which develops progressively, but is of sudden hemodynamic significance. Another common cause of stroke is the abrupt lodging of an embolus within the cerebral circulation and its consequent, distal cerebral tissue ischemia.

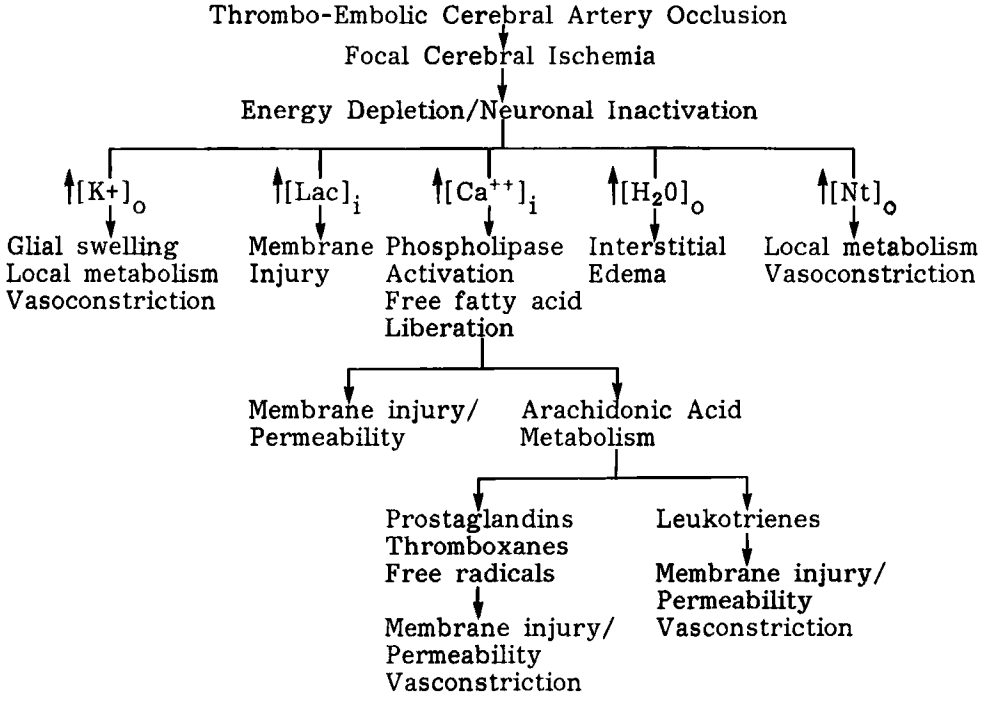
The treatment of acute stroke today consists mainly of individualized management dictated by diagnostic criteria and clinical predictors or outcome.[4] Specific therapy for acute stroke includes normalization of systemic blood pressure, maintenance of vital functions and rehabilitation.[5] The lack of any specific agent of proven benefit in the treatment of acute stroke is exemplified by the recent summation regarding stroke treatment, "single, best therapy: meticulous nursing care."[6]

Although it is commonly accepted that even a brief period of ischemia results in irreversible brain damage, recent experimental studies with animal models have suggested that this is not universally the case. Indeed, studies of experimental stroke in non-human primates have shown that surprisingly long episodes of cerebral ischemia can be tolerated without permanent disability and irreversible cerebral tissue injury,[7,8] and, more importantly, that such tolerance is determined by the duration and degree of ischemia.[9,10,11] Thus, thresholds of focal (localized) cerebral ischemia determine functional cellular activity[12] and cerebral tissue viability.[13] The clinical implications of the threshold concept are that in acute stroke pharmacological intervention which increases residual cerebral blood flow would be expected to lessen tissue injury and improve long-term functional outcome.[14] Alternatively, treatment which enhances the brain's tolerance to ischemic conditions would also be expected to benefit the acute stroke victim.

Animal model studies of brain ischemia have proposed diverse biochemical strategies for pharmacological intervention in acute stroke. Excellent reviews of such studies have been published recently.[15-18] In general, the experimental findings support the pathophysiologic cascade illustrated schematically in Figure 1.

FIGURE 1

ISCHEMIC STROKE: PATHOPHYSIOLOGIC CASCADE



Abbreviations: ↑[]o = increase in the extracellular concentration of potassium (K^+), water (H_2O), and neurotransmitters (Nt); ↑[]i = increase in the intracellular concentration of lactate (Lac) and calcium (Ca++).

Note: Focal Cerebral Ischemia refers to a localized area of reduced blood flow sufficient to impair cerebral tissue function. Arrows indicate increases in or production of the species or event noted and progression of ischemic injury.

From the above simplified scheme, possibilities for pharmacological intervention in acute stroke are suggested. In the section which follows, an attempt has been made to review such possibilities briefly by pharmacological mechanism of action and emphasizing the supportive experimental and clinical data where possible.

MODIFIERS OF CEREBRAL METABOLISM

As is implied in Fig. 1 and might be inferred logically in the treatment of acute stroke, a pharmacological reduction in the ischemic cerebral tissue's metabolic demand might be expected to protect against irreversible injury. Therapies which exemplify this approach are summarized below.

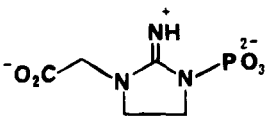
Hypothermia - sufficient to depress brain metabolism has been proposed as beneficial in stroke.[19] Hypothermia also slows cerebral perfusion by increasing blood viscocity and can therefore have detrimental effects.[20]

Barbiturates - such as phenobarbital, have been shown to inhibit cellular glycolysis and hence cerebral metabolism.[21] Although early studies were suggestive of a benefit in the treatment of stroke,[22] cardiorespiratory liability resulting from the high dose levels required have made this a last choice treatment.[23]

Anticonvulsants - have also been proposed as treatments for acute stroke on the basis of their activity as inhibitors of cerebral metabolism.[24] In particular, phenytoin has shown protective effects during experimental cerebral ischemia, but clinical data in acute stroke have not been reported.[25]

ATP Modifiers - The extreme susceptibility of the brain to ischemia results from the very limited reserves of the high energy phosphates, ATP and phosphocreatine. Therapeutic efforts to maintain the levels of these high energy intermediates have taken several paths.

The infusion of ATP itself in dogs has been reported not to benefit ischemic tissue.[26] However, in animal models, the beneficial effect of acute administration of phosphocreatine in combination with creatine phosphokinase[27] and chronic administration of cyclocreatine phosphate (1) has been reported.[28] A number of agents have also been reported



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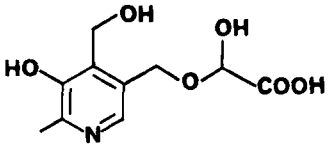

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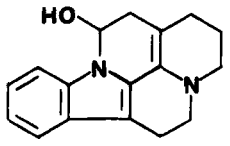
to inhibit the decline of neuronal ATP levels in a hypoxic or ischemic brain. These include nicergoline,[29] pyridoxylate[30] (2), dihydroergotoxine,[31] vindeburnol (RU24722) (3),[32] dihydroergocristine,[33] and recently the substituted quinoline (4).[34] In rat cortex, indoramin (5) at subhypotensive doses increased the rate of ATP synthesis,[31,35] Naftidrofuryl (6) was also shown to both stimulate synthesis and slightly inhibit the utilization of ATP.[31]



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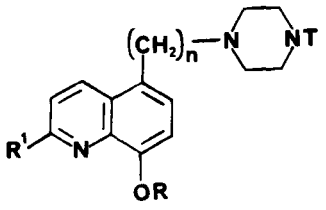

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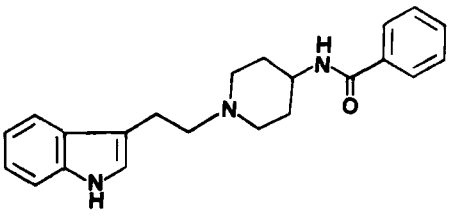

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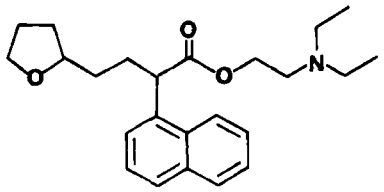

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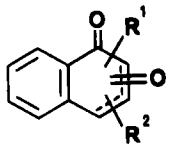
Oxyhemoglobin Destabilizing Agents - The partial pressure of oxygen available in ischemic tissues is influenced by the rate of dissociation of oxygen from hemoglobin.[36] In ischemic tissue, the slowed clearance of the metabolic endproducts, CO_2, and lactic acid combine with endogenous 2,3-diphosphoglycerate to destabilize the oxyhemoglobin complex and make oxygen more readily available to the tissues. Efforts to develop agents which augment this process have also been reported. A series of quinone derivatives, exemplified by (7), have been disclosed as enhancers of blood 2,3-diphosphoglycerate levels.[37]



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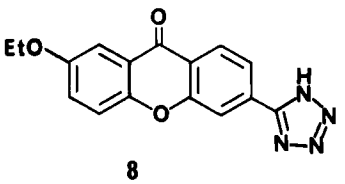

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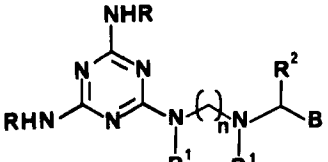

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Although not operating through a modification of diphosphoglycerate levels, xanthone derivatives (8) have been reported, which interact directly with the oxyhemoglobin complex and bring about destabilization.[38]

Substituted triazines (9) have also been claimed to increase the partial pressure of free arterial oxygen.[39]

Blood Substitution - The appeal of this approach is the reduced viscosity and hence easier penetration of the ischemic foci by the oxygenated carrier. The major focus of blood substitution research has been on Fluosol DA, an emulsion of perfluorodecalin and perfluorotripropylamine. Clinical[40] and animal[41,42] studies using Fluosol DA alone or in combination with mannitol[43] have shown some beneficial effect. A related approach to cerebral oxygenation utilizes the perfusion of an oxygenated fluorocarbon nutrient emulsion through the cerebrospinal pathway.[45,45] The salvage of severely ischemic tissue by this technique has been reported recently in a cat model.[46]

The generation of neohemocytes, prepared from purified human hemoglobin and 2,3-diphosphoglycerate encapsulated in a membrane of phospholipid and cholesterol, has been reported.[47] The small particle size and good in vivo toxicological profile in rats, should make this a viable candidate for blood substitution trials in experimental stroke. Additional simplification of this concept, using iron tetraphenylporphyrin complexes has also been disclosed.[48]

CEREBRAL VASODILATORS

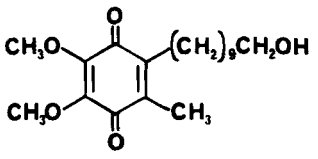
Prompt return of cerebral blood flow would be expected to limit ischemic tissue necrosis. However, nonselective agents may exacerbate ischemic damage either by diverting blood to nonischemic brain areas, the so called 'steal syndrome,'[49,50] or by causing hypotension through peripheral vasodilation.[51] Although beneficial reports of cerebrovasodilators in stroke are uncommon,[51] vincamine and papaverine have been reported active in experimental studies.[52] Cerebrovascular-selective calcium channel antagonists have been

described.[53] Of these flunarizine and nimodipine have been reported to be active in animal models of hypoxia and ischemia.[54,55] Clinical studies using nimodipine in thromboembolic stroke remain inconclusive; however, this agent has shown efficacy in the treatment of subarachnoid hemorrhage-induced cerebral vasospasm.[56]

INHIBITORS OF NEURONAL INJURY

Proposing, as the threshold concept suggests, that an ischemic penumbra or area of compromised yet viable brain may dominate in acute stroke,[57,58] pharmacological intervention to prevent this tissue from progressing to irreversible injury would seem warranted.[59] Several strategies have been proposed, based on experimental studies on cerebral ischemia pathophysiology.

Antioxidants - A significant body of evidence has established that during an ischemic event, free radicals such as superoxide anion, hydroxyl radical, or lipoperoxides are generated by a variety of mechanisms.[60,61,62] Free radical scavengers have been investigated in experimental stroke models. These agents would augment endogenous antioxidant systems. Mannitol,[63] vitamin E (tocopherol)[64], methylprednisolone,[65] ubiquinone, (coenzyme Q),[66] a component of the



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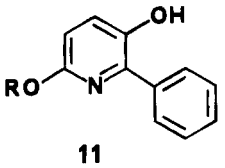

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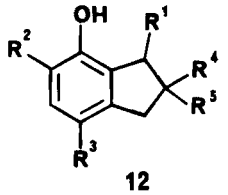

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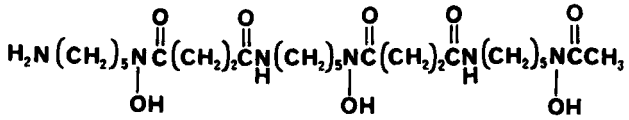
mitochondrial electron transport system, its derivative idebenone (CV 2619) (10),[67] and 1,2-bis(nicotinamide)propane (AVS),[68] have all been reported to function in part as free radical scavengers and inhibit lipid peroxidation. Several phenolic species (11)[69] (12)[70] have also been claimed to act as antioxidants in the treatment of hypoxic conditions.

Iron Chelators - The presence of iron in a wide range of enzymatic systems, such as hemoglobin and ferritin, make free iron readily available under ischemic conditions. This iron is thought to catalyze the generation of free radicals from reactive oxygen species.[71,72] Stroke therapy based on the chelation of free iron has received scant attention. However, a study of deferroxamine (13) in rats has confirmed the potential use of this approach in brain resuscitation.[73]



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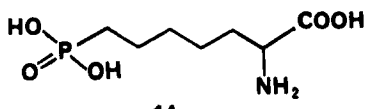
Excitatory Amino Acid Antagonists - Following an ischemic insult, the massive release of glutamate and aspartate from cerebral tissues has been confirmed both in vitro[74] and in vivo.[75,76] These neurotransmitters have been shown to contribute to neuronal cell death possibly through deplorization induced edema.[77]



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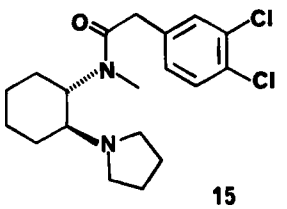
Recent studies using 2-amino-7-phosphono-heptanoic acid (7APH) (14),[78,79] a specific N-methyl-D-aspartate (NMDA) receptor antagonist, have shown that by prior intracerebral administration, a protective effect against hippocampal injury is observed in animal models following a temporary ischemic[80,81] and hypoglycemic[82] insult.

Opiates - Following the observation that the opiate antagonist naloxone could improve the symptoms of stroke in both man[83] and gerbils,[84] many subsequent studies, including clinical trials have appeared which have both supported[85] and refuted[86] these original findings. Later studies have shown that naltrexone[87,88] and levallorphan[89] also exert a protective effect in animal[87] and human trials.[89]

The mechanism by which naloxone and associated opiates exert their protective actions is not known. Stimulation of selected brain motor centers may be responsible for the apparent neurological improvement seen in both animals and in man.[90] Given the benefit of both naloxone and naltrexone in prolonging poststroke long-term survival in cats,[87] the observation that opiate antagonists reverse opiate induced leucocyte chemotaxis[91] and superoxide release[92] may be of relevance.



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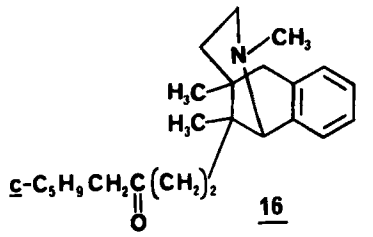

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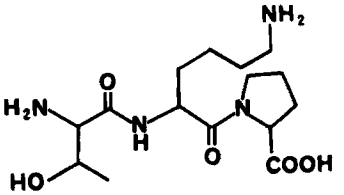
A central role for the Kappa receptor is suggested by recent studies which demonstrate that dynorphin 1-13[93,87,94] and the Kappa agonist U-50,488E (15)[95] afford protection from cerebral ischemia in selected animal models. It is of interest to note that the Kappa antagonist WIN 44,441-3 (16) has also been shown active in improving the neurologic recovery from ischemic spinal cord injury.[96]

Chemotaxis Inhibitors - It has been shown that by four hours following ischemia, leucocytes become concentrated in areas of low blood flow. These recruited leucocytes may, during the critical early hours of ischemia, contribute to the evolution of neuronal damage.[97] Inhibition of leucocyte migration within areas of compromised CNS tissue or inhibition of resulting phagocytic activity might reduce the progression of an initial ischemic insult. Distinct from known inhibitors of arachidonic acid metabolite induced chemotaxis, a tripeptide inhibitor of leucocyte phagocytosis (17) has also been been developed by the Pasteur Institute.[98]



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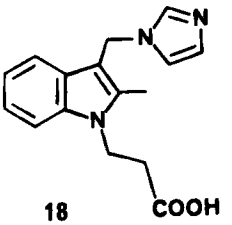
INHIBITORS OF DAMAGING ISCHEMIC METABOLITES

As the schematic pathophysiologic cascade (Fig. 1) suggests, activation and/or release of inhibition of certain metabolic pathways in ischemic tissue can lead to the accumulation of active metabolites which might exacerbate the progression to irreversible injury.

Prostaglandins - can be vasoconstrictive and detrimental ($PGF_{2\alpha}$) or vasodilatory and beneficial (PGI_2) in ischemic tissue.[99] Inhibition of the cyclooxygenase pathway may prevent the accumulation of vasoconstrictive prostaglandins and thromboxanes (TXA_2), and therefore, prevent further ischemia. In general, however, both cyclooxygenase inhibitors[100,101] and prostacyclin[102,103] have shown controversial results in experimental stroke models. Indeed, recent clinical trials with prostacyclin infusions in acute stroke have not been promising.[104] Initial studies with a thromboxane synthetase inhibitor, dazmegrel (UK 38,485) (18), have also been discouraging.[105]



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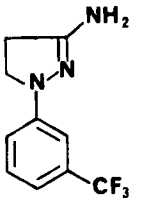
Leukotrienes - are produced from arachidonic acid via the lipoxygenase pathway. These compounds are, in general, vasoconstrictive, frequently increase vascular permeability,[106,107] have recently been shown to accumulate in ischemic brain[108] and may adversely affect outcome from acute stroke. The activity of lipoxygenase inhibitors in stroke models has yet to be reported, however, several compounds, including BW755c (19)[109] and nafazatrom (20)[110] have shown promise in models of myocardial ischemia.



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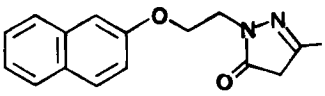

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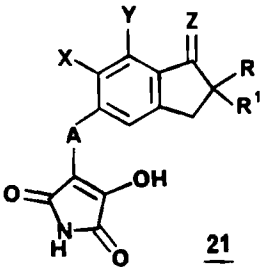
OTHER APPROACHES

Antiedema Agents - have been proposed for some time as an approach to the treatment of acute stroke. Indeed, work with experimental models of stroke has demonstrated the importance of edema in the pathogenesis of ischemic cerebral injury.[112,113] However, steroid and osmotic diuretic therapies have shown inconsistent activity in acute cerebral ischemia.[114,115] A recent report suggests that cyclooxygenase and lipoxygenase inhibitors may



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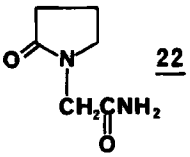
substitute for or be additives to steroid therapy for cerebral edema.[116] A series of cerebral antiedema compounds has recently been reported. (21).[117]

Fibrinolytic Agents - Fibrinolytic removal of a clot with subsequent tissue reperfusion is an appealing solution to the problem of a thromboembolic stroke. However, this approach may lead to hemorrhagic complications. This not withstanding, small clinical studies using the nonselective plasminogen activators, urokinase and, less frequently, streptokinase, have been reported.[118,119] A recent study of the embolus selective tissue plasminogen activator (TPA) in a rabbit embolic stroke model has yielded encouraging results.[120] New heparinoid drugs which lack the bleeding risk of those presently available have also been reported.[121]

Nootropic - or cerebroactive drugs have been reported to be active in animal models of brain hypoxia and ischemia.[112] Limited experimental[123] and clinical[124] data have suggested improvement after treatment with the prototypic nootropic, piracetam (22).



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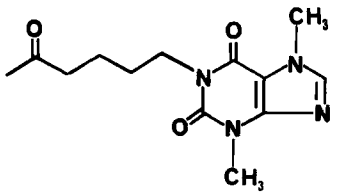
Hemorrheologic Approaches - to the treatment of acute stroke have utilized pharmacological and non-pharmacological means by which to decrease blood viscocity and thereby improve residual cerebral blood flow. Hemodilution has been shown effective as a treatment for acute stroke.[125,126] Pentoxiphylline (23), which improves blood flow by increasing red blood cell deformability,[127] was suggested as a treatment for stroke[128] and is now in clinical trials.



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Enhancers of Neuronal Plasticity - Gangliosides, a class of complex biological molecules which belong to the glycolipid family, have been known for some time to potentiate axonal sprouting after peripheral nerve injury, and more recently such findings have been extended to the CNS.[129] GM1, a specific monosialoganglioside, has been shown to reduce cerebral edema in an animal model of brain injury[130] and to improve neurological outcome in a small clinical trial of stroke patients.[131]

Lactic Acidosis - arising from anaerobic glucose metabolism has been proposed to play a role in ischemic cerebral injury.[132,133] Substitution of glucose by 1,3-butanediol, a ketone substrate, has been reported to be beneficial in stroke models.[134]

CONCLUSION

From the forgoing survey, it is apparent that despite the breadth of pharmacological approaches which have been examined for the treatment of stroke, no single effective therapy has yet emerged. However, from the rapid progress currently being made in the understanding of stroke pathophysiology it is probable that effective strategies for stroke therapy will be identified in the next few years.

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SECTION III — Chemotherapeutic Agents

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Chapter 12. Antimicrobial Drugs — Clinical Problems and Opportunities

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Introduction — Future opportunities for product development in antimicrobial chemotherapy will be guided as much by the economics of therapy as by the perceived need for improved antimicrobial coverage.[1] Current economic trends indicate that incremental improvements in spectrum or potency will not be sufficient to guarantee the profitability of new agents.[1,2,3] Indeed, new antimicrobials will have difficulty getting placed on the hospital formulary unless they demonstrate clear economic and therapeutic advantages.[4-7] A significant advantage would involve the use of some new compounds as single agents, replacing expensive combination therapy with less potent compounds.[8,9] In order to accomplish this, new antimicrobials would have to have an outstanding spectrum, including many of the organisms listed in Table 1, that have proven difficult to treat with currently available agents. The purpose of the current review will be to describe several of these organisms and to discuss their mechanisms of antimicrobial resistance where they are known. Effort will be made to describe new agents that have shown promising activity against these clinical pathogens. The microorganisms reviewed have been chosen based upon the following observed clinical trends: a) drug-resistant, Gram-positive cocci have made a resurgence in causing nosocomial infections; b) infections involving enteric bacilli like *Enterobacter, Serratia* and *Proteus* often remain refractory to therapy with broad spectrum drugs; c) Gram-negative non-fermenters like *Pseudomonas* and *Acinetobacter* remain multi-resistant; and d) atypical mycobacteria and fungi are increasing in frequency in infections involving immunocompromised patients.[5,10,11,12]

Importance of Gram-positive Cocci — Gram-positive cocci continue to be extremely important in causing nosocomial infections in the United States.[10,14] *Staphylococcus aureus* remains an important pathogen in primary bacteremia and with cutaneous and surgical wound infections.[10,13] The importance of *S. epidermidis* is growing as a pathogen in intensive care units and with infections in immunocompromised patients.[10,14,15] The role of the staphylococci in nosocomial infections is not surprising, considering their ubiquitous nature in the environment. From 50 to 90% of hospital personnel are nasal carriers of *S. aureus,* and *S. epidermidis* is present on the skin of >90% of the general population, making the reservoir for infection extremely large.[10,16-19] The outcome of infection is influenced by the overall susceptibility of these organisms to antimicrobial agents. While the majority of staphylococci in the hospital environment are resistant to penicillin due to the elaboration of penicillinase, more alarming trends are occurring with regard to their susceptibility to newer agents.[20,21] Staphylococci as well as other Gram-positive cocci, can demonstrate decreased susceptibility to a wide variety of beta-lactams by either a tolerance mechanism or some intrinsic change such as altered penicillin binding proteins (PBPs). Tolerant organisms, by definition, show a dissociation between the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) to beta-lactams.[20-25] In this sense, tolerance does not represent a form of true resistance since in the tolerant strains examined to date, the MIC has been essentially the same as observed in routinely susceptible isolates.[20] While tolerant strains maintain a normal MIC to beta-lactams, some block exists in the series of events leading to bacterial lysis. This blockade is most likely at the level of release and activation of the cell's autolytic enzymes.[26-28] Several investigators have shown that the detection of tolerance *in vitro* is highly dependent upon the culture and testing conditions employed.[29-31] While tolerance has been identified in over twenty species, the clinical significance of the phenomenon remains uncertain.[32,33]

Table 1. Clinical Pathogens Often Resistant to Antimicrobials

Pathogen	Antimicrobial MIC_{90}[a]				
	Ampicillin	Tobramycin	Cefoxitin	Cefotaxime	Norfloxacin
Staphylococci Meth-R	>64	32	>64	>64	8
Enterococci	4	16	>64	64	16
Pseudomonas aeruginosa	>64	4	>64	64	4
Acinetobacter	>64	16	>64	64	16
Enterobacter cloacae	>64	4	>64	0.5	0.5
Serratia marcescens	>64	16	64	0.5	4

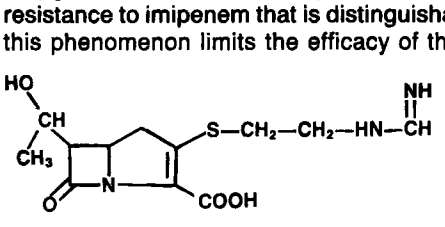
[a]Minimal inhibitory concentration required to inhibit growth of 90% of isolates, from ref. 168.

A subject generating much greater concern in the clinic, involves the potential spread of methicillin-resistant staphylococci (MRS). High-level methicillin resistance (MR) can be rapidly-distinguished from the tolerance phenomenon, in that MR is associated with high-level resistance to semisynthetic penicillins and cephalosporins. The incidence of MRS in the United States has been highly variable between institutions, being highest in large teaching hospitals.[5] From a few to 50% of *S. aureus* infections are caused by methicillin-resistant isolates.[34-37] The frequency of *S. epidermidis* isolates that are MR is reported to be even higher.[5,38,39] As might be expected, many patients infected with MRS are immunocompromised in some way and are least able to resolve the infection, necessitating aggressive antimicrobial therapy.[5,10,40] Since many MRS are also resistant to aminoglycosides, therapy usually consists of vancomycin.[10] With the suggestion of an increase in the frequency of MRS in some institutions and the limited choice of therapy currently available, attention has turned to developing safe and effective agents against these pathogens. In order to evaluate the clinical need for developing such effective new agents, a full understanding of the mechanism and potential for further spread of this resistance must be considered.

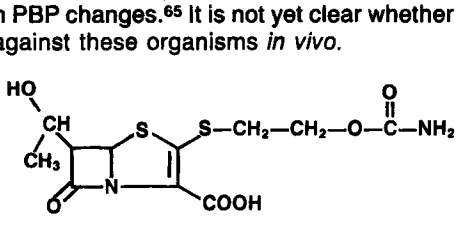
Evidence suggests that the overall incidence of nosocomial infection with staphylococci may not actually increase in institutions where MRS have become endemic.[34] MRS strains may simply replace endemic strains that are susceptible to methicillin in the institution.[34] Such selective pressure is likely due to the increased use of beta-lactamase stable penicillins and cephalosporins occurring in contemporary clinical practice. The magnitude of the problem can best be predicted from the mechanism of MR which has been recently elucidated. Resistance to methicillin is intrinsic and is not due to the production of penicillinase.[41] Some confusion has arisen in the literature concerning the role of the penicillinase plasmid in MR. It was recognized early that the *in vitro* transduction to MR in *S. aureus* was dependent upon the recipient strain containing a penicillinase plasmid.[42] This increased frequency of transduction to MR could also be accomplished if the recipient was previously lysogenized by prophage.[43] This dependence upon the penicillinase plasmid for successful conversion to MR is misleading since the structural *mec* gene is believed to be located on the chromosome in *S. aureus*.[42] This dependence on the penicillinase plasmid for establishment and expression of MR in *S. aureus* laboratory isolates may have some relevance in clinical isolates as well. Although the majority of resistant strains in one study contained the penicillinase plasmid, in 80% of the clinical strains studied the plasmid could be eliminated from the isolates and MR was maintained.[44] It is clear that MR is not due to elaboration of penicillinase and the *mec* gene is not located on plasmids encoding penicillinase. However, the presence of these plasmids and/or prophage may play

some role in making a methicillin susceptible cell accept the *mec* determinant.[43,44] Studies are in progress to identify the locus of the *mec* determinant on the *S. aureus* chromosome and study the nature of its transfer in this species.[45] Since the spread of MR is not due directly to an R factor, an understanding of the expression of this intrinsic resistance plays an important role in predicting its potential clinical impact. MR is a heterogeneous form of resistance in most strains in that under normal *in vitro* culture conditions of 37°C, only a portion of the population expresses resistance.[44] The percentage of cells that are resistant can be raised to nearly 100% under culture conditions of 30°C or in medium containing 5% NaCl.[45-49] The heterogeneous nature of MR is a stable trait since resistant populations will return to a mixed phenotype when cultured under conditions that do not favor expression of the resistance.[44] Consistent with this phenomenon has been the finding that expression of low affinity PBPs correlates with the occurrence of MR. Beta-lactam susceptible strains of *S. aureus* and *S. epidermidis* normally contain four PBPs that can be detected in polyacrylamide gels, following labelling of membrane preparations with ^{14}C-penicillin G.[55] It is believed that PBPs 2 and 3 are the essential binding proteins in *S. aureus* that are responsible for cell growth.[55] It is believed that PBPs 2 and 3 are the essential binding proteins in *S. aureus* that are responsible for cell growth.[55] Resistant strains have been found that contain variants of these lethal proteins and these resistance-associated PBPs have low affinities for methicillin and many other beta-lactams.[44,45,53,54,57,58] The low affinity PBP most commonly identified in *S. aureus* has a molecular weight of 78,000 daltons and migrates on SDS polyacrylamide gels very close to PBP-2[44,53] Labelling studies using whole cells indicated that the low affinity protein PBP-2′ was not saturated until levels of methicillin reached 1 mg/ml, which correlated with a similarly high MIC to the drug.[54] The "normal" PBPs in such a resistant cell are saturated at concentrations up to 1,000-fold below this level, therefore, PBP-2′ apparently can act independently in carrying out all the necessary functions associated with normal cell division.[54] As would be predicted, culturing the resistant *S. aureus* cells at pH 5.2 both eliminated expression of PBP-2′ and methicillin resistance.[54] This mechanism of intrinsic resistance seems even more complex in that both individual PBPs other than PBP-2′ and multiple changes in several PBPs have been associated with resistance to beta-lactams.[53,59] Beta-lactams have also been shown to be effective inducers of high levels of the low affinity PBPs.[59,60] Thus, in strains of staphylococci, there are low affinity PBPs that are responsible for conferring intrinsic resistance to a wide variety of structurally diverse beta-lactams. Given that some beta-lactams have been shown to induce higher levels of these low affinity PBPs in the cell, is there a rationale for discovering new beta-lactams that are effective against these nosocomial pathogens?

A number of new beta-lactams have been developed that show improved activity against MRS, presumably due to their increased affinity for the resistance associated PBPs. Cefmetazole is a broad spectrum, cephamycin-type antibiotic with cyanomethylthioacetamide and methyltetrazolylthiomethyl radicals on the 7 and 3 positions, respectively (Table 2). Although some degree of activity is claimed against MRS, studies with PBPs of these organisms indicate that the low affinity PBP-2′ is saturated by cefmetazole levels one hundred-fold above the MIC.[61] A similar minor improvement in *in vitro* potency against MRS is seen with imipenem (N-formimidoyl thienamycin (1), in which published MIC_{90} values generally range between 8 and 64 μg/ml.[62-64] Strains of *S. epidermidis* have been shown to express an inducible form of resistance to imipenem that is distinguishable from PBP changes.[65] It is not yet clear whether this phenomenon limits the efficacy of the drug against these organisms *in vivo*.



1



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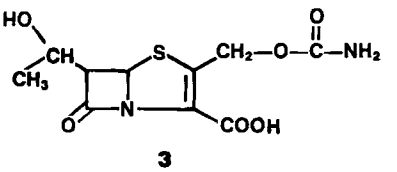


3

Certain members of the penem class of antibiotics have shown promising activity against MRS. While sulfur-linked compounds at C-2 of the penem nucleus like SCH-34,343 (2) have poor activity against MRS, some carbon-linked compounds demonstrate significantly improved potency *in vitro*.[64,66,67] As shown in Table 2, the reported MIC_{90} values for FCE-22,101 (3) against MRS are ≤8 μg/ml.[66,67] The effect of linking the side chain to the penem nucleus through a carbon rather than sulfur, is interesting when comparing the *in vitro* activity of penems SCH-34,343 and FCE-22,101 against MRS (Table 2). Other C-2 carbon-linked penems in development have shown similar increases in potency against these organisms.[68,69] Studies describing the relative affinities of these new penems for the PBPs in MRS would be helpful in describing the structural specificity required for relevant activity. The impact of intrinsic beta-lactam resistance in Gram-positive organisms should not be underestimated. In addition to MRS, a number of streptococci have demonstrated the presence of low affinity PBPs that have been associated with beta-lactam resistance.[56,58,86-90] The presence of these low affinity or slow reacting PBPs in strains of Enterococci accounts for the low activity of many newer beta-lactams against problem pathogens like *S. faecalis* (Table 1).[58,87,89,91,92] Thus, new opportunities for discovering broad spectrum antimicrobials that show real improvements in efficacy against problem Gram-positive organisms may logically involve compounds other than beta-lactams.

Indeed, at least two non-beta-lactam approaches offer promise for obtaining highly active compounds against MRS. While mortality associated with infections caused by MRS can be extremely high, vancomycin has been established as the effective drug of choice.[70,74] In an effort to find glycopeptides with improved pharmacologic characteristics compared to vancomycin, a number of analogs have been studied. Teicoplanin is currently in development and demonstrates improved pharmacokinetics with similar activity to vancomycin against MRS.[75-77] Quinolones represent one of the more promising classes of compounds that possess broad-spectrum antibacterial activity including MRS.[78-82] While the *in vitro* activity of the first of the newer quinolones, norfloxacin, is not great against MRS, ciprofloxacin MIC_{90} values are equivalent to those of vancomycin against these organisms (Table 2).[78,80,83,84] Pefloxacin also has excellent activity against MRS *in vitro* and has been shown to be equally effective as vancomycin in treating experimental endocarditis with these organisms in rabbits.[85] The impressive activity of some quinolones against MR organisms lies in their mechanism of action which avoids the PBP targets that confer intrinsic resistance.[80] A chapter detailing recent developments in the quinolone area appears later in this volume.

Table 2. Activity of Antimicrobials against Methicillin-Resistant *S. aureus*

Antimicrobial	MIC_{90}[a]
Cefmetazole	16
Imipenem	16
SCH-34,343	>64
FCE-22,101	8
Teichoplanin	0.5
Ciprofloxacin	0.25

[a]Minimal inhibitory concentration required to inhibit 90% of strains, from refs. 61, 67, 77, and 78.

Improved Therapy for Treating Infections with Gram-negative Bacilli

Enterobacteriaceae are still the causative pathogens in over 50% of all nosocomial infections in the United States.[10] Despite the extensive effort to develop efficacious antimicrobials for use against this group, several species still represent significant therapeutic challenges with currently available compounds (Table 1). In addition, current trends indicate an increased occurrence of polymicrobial infections in hospitalized patients, further challenging the efficacy of existing antimicrobial agents.[11,93] One study has shown that one-third of nosocomial bloodstream infections from patients in a cancer treatment center were polymicrobial.[94] The diversity of resistance mechanisms to antimicrobial agents within this family of organisms has greatly affected the development of more effective agents (Table 3). The efficacy of aminoglycosides, beta-lactams, and chloramphenicol has been limited by the frequent presence of modifying or inactivating enzymes present in Gram-negative organisms.[95-99] This problem has seriously limited the clinical utility of many agents, resulting from the spread of plasmid-encoded enzymes. Species-specific beta-lactamases encoded on the chromosome have been implicated in the resistance to many newer beta-lactams by hydrolytic and non-hydrolytic mechanisms.[100-106] In addition, mechanisms of reduced permeability across the outer membrane and extrusion of intracellular drug, has also limited the usefulness of some beta-lactams and the tetracyclines.[107-110] More recently described mechanisms of resistance have involved the appearance of low affinity PBPs that have reduced the effectiveness of many beta-lactams.[111,112]

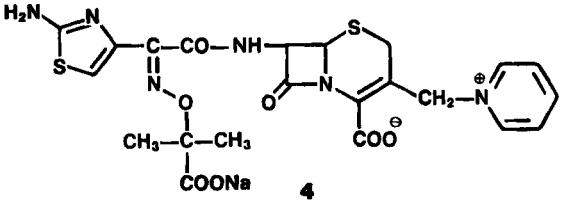
The development of the third generation cephalosporins was in part, a response to organisms that possessed many of these resistance mechanisms. While these antimicrobials demonstrate improved activity against many Gram-negative organisms, some isolates (Table 1) are often resistant. In the 1982 National Nosocomial Infections Study >15% of *Serratia marcescens* reviewed were resistant to cefotaxime and >8% were resistant to moxalactam.[13] Chromosomal beta-lactamases have been found which can inactivate cefotaxime, ceftazidime, moxalactam, and monobactams.[113-115] Resistance emergence during therapy with cephalosporins has also been a potential problem in infections caused by isolates of *Enterobacter cloacae, Citrobacter freundii,* and indole-positive *Proteus* sp.[116-117] This potential for resistance emergence lowers the economic impact of the newer cephalosporins since they require combination with aminoglycosides in some clinical settings.[10] Development of enhanced potency against Gram-negatives has often compromised the Gram-positive activity of cephalosporins, which has allowed superinfections with organisms like *Streptococcus faecalis.*[118,119]

Table 3. Mechanisms of Antimicrobial Resistance among the *Enterobacteriaceae*[a]

Antimicrobial Class	*Mechanism*
Beta-lactams	**Inactivation by beta-lactamase, decreased permeability across outer membrane, presence of low affinity PBPs.**
Aminoglycosides	**Modification by N-acetylation, O-phosphorylation, or N-nucleotidylation. Failure to enter cell. Altered 30s ribosome.**
Chloramphenicol	**O-acetylation**
Tetracyclines	**Transport out of cell**
Sulfonamides	**Altered dihydrofolate synthetase**

[a]Adapted from ref. 40.

The problems associated with finding efficacious agents against enteric organisms are magnified greatly for *Pseudomonas aeruginosa* and some other nonfermenting Gram-negatives. The difficulty of finding potent anti-pseudomonal agents involves both the virulence and physiology of this increasingly significant pathogen. Currently *P. aeruginosa* is responsible for causing about 12% of all reported infections in community hospitals in the United States.[20] Data from the Centers for Disease Control indicates that 10% of urinary tract infections, 9% of surgical wound infections, 17% of lower respiratory tract infections, and 11% of bacteremias are caused by this organism.[120] Particularly susceptible patients are those with burn wounds, cystic fibrosis, acute leukemia, organ transplants, and intravenous-drug abusers.[120-123] Polymicrobial infections involving *P. aeruginosa* are particularly difficult to treat with mortality rates from one study equaling 57% compared to 48% observed with polymicrobial infections with Gram-negatives excluding *Pseudomonas.*[121,124] The difficulty associated with the treatment of this organism involves the extensive armament of inactivating enzymes, restricted outer membrane permeability, and alterations of lethal targets for antimicrobials, a subject reviewed previously in this and other publications.[102,125] Promising new compounds for use against this organism have included a number of third generation cephalosporins, carbapenems, monobactams, and quinolones. Ceftazidime (4) is an aminothiazolyl cephalosporin with excellent *in vitro* potency against many Gram-negatives including *P. aeruginosa.*[126,127] Ceftazidime is highly stable to hydrolysis by almost all beta-lactamases due to the propylcarboxy side chain and is also a weak inducer of Type Id beta-lactamase of *P. aeruginosa.*[128,129] It also binds less well to Type I enzyme and is, therefore, a less effective inhibitor of this enzyme.[129] Ceftazidime binds primarily to PBPs 3 and 1a in *E. coli* and *P. aeruginosa* with little affinity for PBP-2 and the non-lethal targets, PBPs 4-6.[129] This "lethal site directed" activity along with its avoidance of interaction with beta-lactamase, explains why ceftazidime is significantly more active against *P. aeruginosa* than many other cephalosporins.[126,127] Ceftazidime has been shown to be highly effective in treating serious infections, although its moderate Gram-positive spectrum necessitates use of combination therapy in several situations.[131,132] The lack of balance in the spectrum of ceftazidime results from its low affinity for the lethal PBPs of organisms like *S. aureus, S. faecalis, and S. faecium.*[133] Other approaches aimed at finding potent anti-pseudomonal compounds have involved the monobactams, possessing a limited spectrum consisting of Gram-negatives and the narrow spectrum antipseudomonal agent cefsulodin.[134,135] The narrow Gram-negative spectrum of these agents results from their restricted PBP binding pattern which includes PBP-3 for aztreonam and PBPs 1A/1B for cefsulodin.[135]



4

Carbapenems and quinolones have shown promise as broad-spectrum antibacterials with potency against Gram-positives and Gram-negatives including *P. aeruginosa.*[136,137] The rationale for pursuing carbapenems as truly broad-spectrum agents, involves their rapid penetration into Gram-negative cells, high stability to essentially all beta-lactamases, and their high affinity for the lethal PBPs in many bacteria.[102,138] Imipenem is the leading example of the class and has proven to be highly efficacious for a wide variety of infections although its use as a single agent against *Pseudomonas* may be limited due to development of resistance.[139,140] Further improvements in the carbapenem area would likely involve development of analogs with higher *in vivo* stability to mammalian renal dipeptidase that would eliminate the need for co-administration of an inhibitor of this enzyme for clinical use of these compounds.[141] The development of quinolone antibacterials is, in part, due to their potential as potent, broad-spectrum agents that would avoid the resistance mechanisms described above. Quinolones are potent bactericidal agents that appear to inhibit bacterial DNA synthesis by inhibiting the activity of DNA gyrase (topoisomerase II), which is responsible for ATP-dependent negative supercoiling of DNA.[142-145] No known bacterial enzymes exist that inactivate quinolones and single step mutations leading to high level permeability resistance occurs only at low frequency

($<10^{-9}$).[146] Mutations can occur to nalidixic or oxolinic acid in the DNA gyrase subunit A and this can decrease the potency of the newer fluoroquinolones.[147,148] The impact of the fluoroquinolones lies in their potential to achieve a balanced Gram-positive and Gram-negative spectrum with improved activity against organisms like MRS, *Acinetobacter* spp., and *P. aeruginosa*.[80]

Increased Clinical Importance of Atypical Mycobacteria

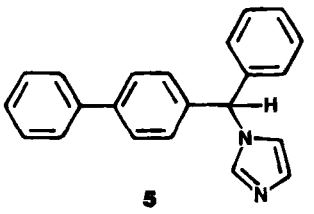
With the decreasing occurrence of *Mycobacterium tuberculosis* infections in the United States, an increase in the clinical significance of the atypical mycobacteria has occurred.[5] These organisms are ubiquitous in nature and have played an increasingly important role in infections in immuno-compromised patients, and in infections associated with invasive procedures such as corneal grafts, prosthetic heart valves, vascular grafts, and breast implants.[5,10,146-148] Isolates of the *Mycobacterium avium/intracellulare* complex have been isolated from patients with acquired immune deficiency syndrome (AIDS), often demonstrating disseminated infection with these organisms.[5,149] The atypical mycobacteria are notoriously resistant to most current antimicrobials including isoniazid and streptomycin, necessitating combination therapy with several agents.[5,150] While imipenem and ciprofloxacin have shown some activity against these organisms, new agents are clearly needed.[151,152] Studies with ciprofloxacin indicate that it is active against *M. tuberculosis, M. fortuitum, M. kansasii,* and *M. marinum* with MICs of 0.78 to 1.56 μg/ml.[153] Ciprofloxacin was not highly active against isolates of *M. avium/intracellulare* and *M. chelonei* however, since MICs were ≥12.5 μg/ml.[153] Since these non-tuberculosis mycobacteria are likely markedly underdiagnosed and since immunocompromised patients will continue to be a growing segment of hospitalized patients, development of new and more effective antimicrobials is warranted.[154]

Need for Improved Antifungal Therapy

Significant improvements in antifungal therapy have been few since the introduction of amphoteracin B in the early 1950's. This lag behind antibacterial therapy is due to a poorer understanding of fungal cell physiology, organisms which like man, are eukaryotes containing a well defined plasma membrane.[157] Further, the causative role of fungi in many infectious disease processes has been poorly understood due to a lack of appropriate diagnostic and culture methods for these opportunistic pathogens. Since fungi are ubiquitous, the presence of these organisms in clinical culture has often been difficult to interpret. Several recognized clinical trends highlight the need for new antifungals with improved efficacy and safety. A number of common hospital procedures predispose patients to opportunistic fungal infections.[5,11] The increased use of indwelling catheters to administer nutrition and medication increases the frequency of fungemia.[5,11] Fungal infections are markedly increased in frequency with immunocompromised patients, particularly those given broad-spectrum antimicrobial therapy.[5] In one medical center, from 25 to 50% of patients with hematologic malignancies were shown to have documented fungal infections at autopsy.[155] Like some other infectious diseases, fungal infections are undoubtedly under diagnosed.[11,155] As methods of culture and diagnosis in the area improve, the extent of morbidity and mortality from opportunistic fungal infections will become clearer. Such advances will also allow the design of better clinical studies by which to objectively measure the efficacy of new antifungals.[5]

The areas in need of improvement for antifungal agents are several. Since many fungal infections are chronic in nature, the introduction of new, broad spectrum antifungals would be a welcomed advance.[5] Much work has been done with the azole derivatives in order to find new oral agents and ketoconazole has been found to be effective in several fungal diseases.[156,157] Ketoconazole has been shown to have decreased side effects compared to amphoteracin B, but the efficacy of ketoconazole is not greater than amphoteracin B.[156] A number of new azole-based antifungals are in various stages of testing and many have shown increased potency *in vitro* and improved efficacy in animal infection studies compared to ketoconazole.[156] Bifonazole (**5**) has been shown to have good *in vitro* activity against dermatophytes compared with miconazole and clotrimazole[158] Two triazole-based compounds Bay-n-7133 (**6**) and itraconazole (**7**) showed good *in vitro* activity against a wide variety of

fungi and were orally active in mouse protection studies.[156] Zinoconazole (**8**) and terconazole (**9**) also showed good activity as broad spectrum antifungals, although, little *in vivo* work has been published on these and other new azole derivatives.[156] The importance of extensive *in vivo* testing of new agents is demonstrated by studies describing UK-49,858 (**10**) a new oral bis-triazole antifungal.[159] UK-49,858 is less active than miconazole or ketoconazole *in vitro* but was an order of magnitude more efficacious than these agents in treating mice with vaginal candidiasis.[160] UK-49,858 also appears to be selective for inhibiting fungal sterol biosynthesis compared with mammalian steroidogenic enzymes.[161] New agents outside of the azole-based class include SF-86,327 from the naftifine series and tioxaprofen, a compound discovered as a non-steroidal anti-inflammatory agent.[156] Tioxaprofen has activity against dermatophytes and *C. albicans* and acts by uncoupling mitochondrial respiration.[156]



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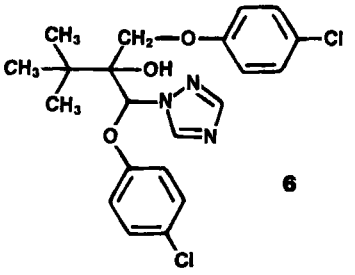

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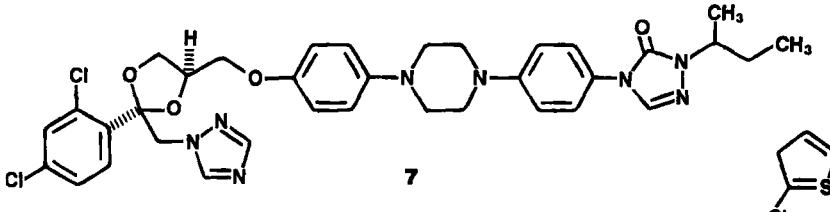

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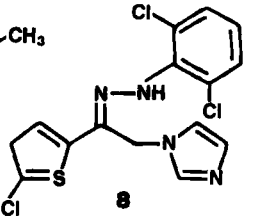

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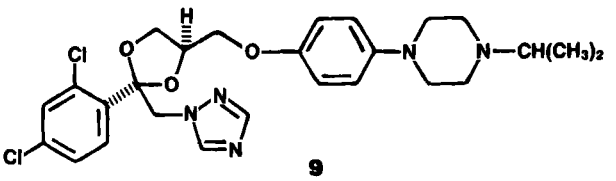

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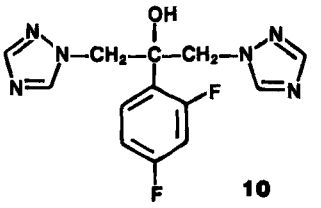

10

Concomitant with the search for new, more potent antifungals, has been the development of novel drug delivery systems aimed at optimizing the therapeutic potential of these agents. Delivery systems involving lipid vesicles (liposomes) have shown promise since they are simple to prepare, biodegradable, and possess no appreciable toxicity.[162,163] Changing the lipid composition of liposomes alters their pharmacokinetics, enabling a more targeted delivery of the chemotherapeutic agent enclosed.[163] While liposomes have been employed as carriers for various chemotherapeutic agents, enzymes, and immunostimulants, recent studies have evaluated their potential as a delivery system for amphoteracin B.[162] Studies in mice indicated that liposome-encapsulated amphoteracin B was as efficacious as free drug in the treatment of and prophylaxis against *Candida albicans.*[162] The maximum tolerated dose of encapsulated drug was over ten-fold higher than that observed for free amphoteracin B.[162] Toxicity observed with the free drug was absent from the liposomal form.[162] The lack of toxicity associated with giving amphoteracin B in liposomes allowed higher concentrations of drug to be administered, producing improved cure rates *in vivo.*[162] Similar results were observed in prophylactic studies using neutropenic mice infected with *C. albicans.*[164] Studies in animals, therefore, suggested that an improved therapeutic index could be achieved by administering amphoteracin B in

liposomes. Limited studies in man further support this conclusion.[165] Significant clinical improvement was observed using liposomal amphoteracin B in eight out of twelve patients with hematologic malignancies complicated by fungal infections. Unlike therapy with free drug, few side effects were observed with drug administered in liposomes.[165] Thus the development of a novel delivery system for amphoteracin B shows promise in mitigating the toxicity associated with this highly effective antifungal. Improved efficacy of the drug may also be realized through the liposome system.[166] Studies with radiolabeled liposomes indicated that they distribute to a large extent in organs rich in reticuloendothelial cells such as the liver, lung, spleen, and bone marrow. These organs are often involved in disseminated fungal infections and more effective levels of drug may be targeted to these infected sites.[165] Secondly, due to the higher potential safety of liposomal amphoteracin B, higher doses might be given in order to achieve earlier clinical improvement. The advantage of giving amphoteracin B in patients pre-disposed to serious fungal infections before overt disease is noted, has been recently documented.[167] The routine administration of a non-toxic dosage form would make this practice more feasible.

Conclusion — New opportunities in antimicrobial chemotherapy will likely involve the development of compounds that avoid the wide variety of microbial resistance mechanisms, while at the same time offering dosing characteristics that reduce therapy costs. Clinical isolates of MRS, *Enterococci, P. aeruginosa, Acinetobacter,* and some *Enterobacteriaceae* are commonly resistant to many aminoglycosides, penicillins, and cephalosporins. New antimicrobials that will be in a position to respond to future clinical needs will be those with high activity against these organisms as well as against the multitude of other pathogens that are currently more susceptible to existing agents. A balanced spectrum including high activity against both Gram-positive and Gram-negative organisms, will enable such drugs to be used as monotherapy, further reducing high therapy costs incurred from the widespread use of antibiotic combinations. Carbapenems and quinolones, perhaps have the greatest potential for producing new antimicrobials possessing such a balanced spectrum. These classes are unaffected by the vast majority of inactivating enzymes and intrinsic resistance mechanisms found in bacteria that have limited the usefulness of other antimicrobials. New agents are greatly needed for treating immunocompromised patients infected with opportunistic pathogens like atypical mycobacteria and fungi. New opportunities in this area may well involve the development of novel approaches designed to more efficiently deliver therapeutic agents to infected tissues. This is currently being tested by administering liposomes that deliver effective levels of amphoteracin B, while at the same time decreasing the toxicity of the compound to host cells.

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Chapter 13. ß-Lactam Antibiotics

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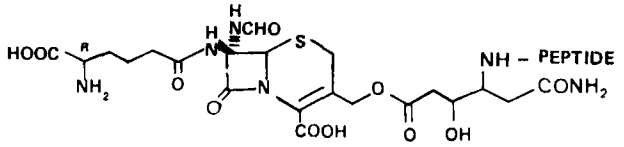
Introduction - In 1985, though progress on the discovery and development of new ß-lactam antibiotics continued, few significantly new and novel structures were described. A major concern in the clinical application of the ß-lactam antibiotics continues to be the development of resistance to these antibiotics particularly by Pseudomonas and Enterobacter species. Reviews have appeared discussing the determinants of microbial resistance to the ß-lactam antibiotics,[1-6] the evolution of resistance to the penicillins and cephalosporins,[7] the role of ß-lactamases,[8-10] PBPs,[11] and permeability barriers.[12-14] One review[15] focused entirely on the mechanism of resistance in P. aeruginosa. A journal supplement was published[16] containing reviews on peptidoglycan biosynthesis and inhibition, the basic design of cephalosporins, structural requirements of effective semisynthetic ß-lactams of all classes, as well as other topics. Reviews also appeared describing new prospects for research on cephalosporins,[17] SAR studies of the cephalosporins,[18,19] comparisons of second generation[20] and third generation cephalosporins,[21] the clinical pharmacokinetics of third generation cephalosporins[22] and one devoted solely to ceftazidime.[23] The proceedings of the 13th International Congress of Chemotherapy which covers all classes of ß-lactam antibiotics was published in two volumes.[24,25] Another review focused on antibiotics, including ß-lactams, used for P. aeruginosa infections.[26] Several reviews were published on the discovery, development and biological profile of imipenem.[27-31] The proceedings of a symposium on carbapenems also appeared.[32] The history and development of the monobactams was reviewed[33] and the proceedings of a symposium on aztreonam was published.[34] Reviews also were published describing the mode of action of ß-lactam antibiotics[35-38], the molecular basis of ß-lactam catalysis,[39] peptidoglycan biosynthesis,[40] PBPs,[41] and the oral absorption of esters of ß-lactam antibiotics.[42] An approach to screening for ß-lactam antibiotics in nature was reported[43]. One study described the interactions of formylamino- and methoxy-substituted ß-lactams with ß-lactamases,[44] while another reported on the leaving group effect in ß-lactam ring opening of the cephalosporins.[45]



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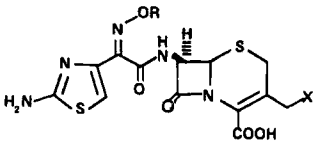
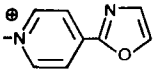
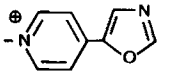
Cephalosporins - The first examples of 7-α-methoxycephem antibiotics to be isolated from bacteria, the cephabacins M(1), were reported by a research group

from Takeda.[45,46] The unique structural feature of these cephems, like the cephabacins F and H described in 1984, is the presence of an oligopeptide side chain at the 3-position. These antibiotics exhibit weak antibacterial activity with properties between those of the cephabacins F and H. A number of new parenteral cephalosporins were reported with improved in vitro activity against Pseudomonas species. Commonly these antipseudomonal compounds were characterized by the presence of a quaternary ammonium function at the 3'-position along with an aminothiazolyloximino grouping in the 7-acyl side chain. Two derivatives, DQ-2522(2) and DQ-2556(3), showed broad spectrum activity including P. aeruginosa with improved potency versus S. aureus (MIC 1.56μ g/ml).[47] Extensive SAR studies on a series of 3-isoindolinium cephalosporins[48] led to the selection of BO-1232(4) and BO-1236(5) for more extensive evaluation.[49] The latter compounds were about ten times more active than ceftazidime against P. aeruginosa and more active against other Pseudomonads as well. Another series of 3'-quaternary ammonium cephalosporins was reported.[50] In this case the heterocyclic groups were pyridothiazoles, pyridoimidazoles, pyridooxazoles and thiazoles. Many of these derivatives displayed potent antipseudomonal activity. MI-4646(6), an aminothiazolyloximino cephalosporin, was reported to show broad spectrum antibacterial activity, including P. aeruginosa.[51] SAR studies on the terminal D-amino acid moiety of the novel cephamycin MT-141(7) showed that conversion to the side chain amide, decarboxylation, acetylation or inversion to the L-configuration led to a decrease in activity against gram-negative bacteria.[52,53] Though it was inactive against P. aeruginosa CL-118523(8) exhibited activity against gram-negative organisms similar to that of ceftazidime and cefotaxime.[54] SAR studies were described for 1-oxacephems related to 6315-S[55], ceftizoxime analogs[56] and heteroacylacetamidocephalosporins.[57] Three publications appeared describing extensive SAR studies on 7-arylglycine derivatives of 7-aminodeacetoxycephalosporanic acid.[58,60] These compounds were orally active and displayed good activity against gram-positive bacteria. The benzothienyl analog LY164846 (9), has undergone extensive biological evaluation.[61] Also studied was a series of 7-arylglycyl cephalosporins (10) having ether substituents at the 3-position.[62] In

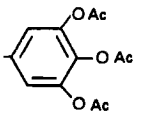


2 R = $-CH_3$ X = 

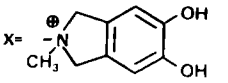
3 R = $-CH_3$ X = 

4 R= $-C_2H_5$

5 R= $-CH_3$

6 R= 

8 R= $-CH_3$

X= 

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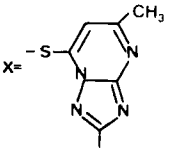
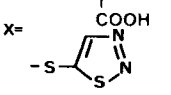
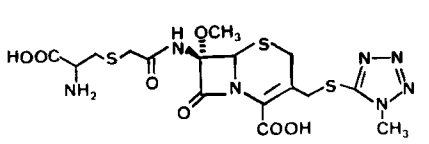
X= 

X= 



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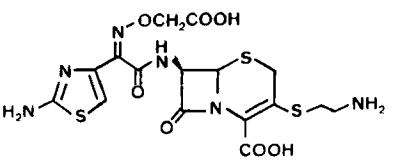

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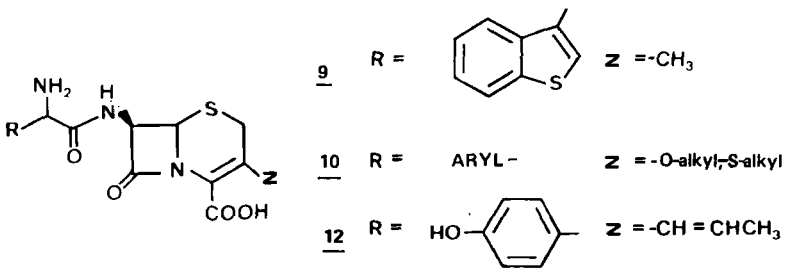
general, increased aliphatic chain length of the ether resulted in increased gram-positive but decreased gram-negative activities. Also, increased chain length led to decreased oral absorption, though sometimes results were mixed. CGP 33098A (11), another new orally active compound, has a spectrum of activity similar to that of FK027 but may be more readily absorbed.[63] Only 20-25% was excreted in the urine of rodents. The p-hydroxyphenylglycyl derivative BMY 28100 (12) showed in vitro activity comparable to that of cefaclor and superior to cephalexin's.[64] However, it was more susceptible to β-lactamases. Pro-drug forms of several parenterally active cephalosporins have been reported. KY-109, a bifunctional pro-drug was well-absorbed orally[65] as were the pivaloyloxymethyl esters Ro15-8075[66-68] and ME 1207.[69,70] Additional publications appeared on the pharmacokinetic and antibacterial properties of the pro-drug cefuroxime axetil.[71] Laboratory or clinical studies also were published on cefixime (FK027),[72] DN-9550,[73] YM-13115,[74,75] FCE 20635,[76] L-105,[77] SK&F 88070,[78] BMY-28142,[79-81] cefpiramide,[82,83] and cefpirome.[84,85]

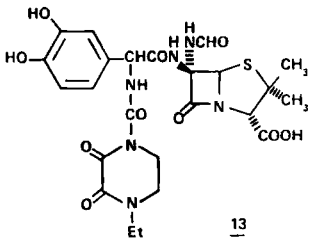


Penicillins - Few reports of new penicillins appeared in 1985. Interest has continued in the novel and potent 6α-formamidopenicillin BRL36650 (13).[86] Two synthetic chemical publications reported the introduction of the 6α-formamido function into the penicillin molecule.[87,88] A new, broad spectrum penicillin VX-VC 43 was reported to be superior to piperacillin, but was not stable to β-lactamases.[89] Comparison of the antimicrobial properties of temocillin side-chain epimers revealed that the R-isomer is twice as potent as the S-isomer against P. aeruginosa, however, epimerization takes place rapidly even under normal bioassay conditions.[90] One review of the in vitro antibacterial activities of temocillin appeared.[91]

Penems - The synthesis and antibacterial properties of a series of novel penems typified by compound (14) was reported.[92] This compound displayed a broad spectrum of activity against both gram-positive and gram-negative bacteria including B. fragilis, but not Pseudomonas. The novel penem, FCE 22101 (15), inhibited the growth of most Enterobacteriaceae but was less active than cefotaxime and ceftazidime against these organisms.[93] Reviews appeared on the antibacterial activity of a series of 2-(substitutedthiomethyl) penems,[94] and on the potent, broad spectrum penem Sch 34343.[95-97]

Carbapenems - The synthesis of several new carbapenem antibiotics structurally related to thienamycin was described including the novel 9-methoxythienamycin (16).[98] These derivatives, however, were significantly less active than thienamycin in vitro. Several additional members of the pluracidomycin family of

carbapenems were isolated from Streptomyces.[99] Pluracidomycin A_2 (17) was reported to be the first example of a sulfinic acid isolated as a microbial metabolite. Mutual pro-drug forms (esters) of the olivanic acids MM22382 and MM13902 with a renal dehydropeptidase I inhibitor were reported.[100] Though urinary recoveries of these derivatives in the mouse were about six times higher than those of the free acids, recoveries still were low (1.8% and 8%). The in vitro activity and ß-lactamase stability of the carbapenem RS-533 was described.[101] Neu has reviewed the special properties of imipenem that contribute to its potent bioactivity, e.g. lack of a permeability barrier, high affinity for key PBPs and resistance to degradation by ß-lactamases.[102] Total syntheses were published for 1α- and 1ß-methylthienamycin,[103] thienamycin,[104] (+) PS-5[105] and the carpetimycins.[106]



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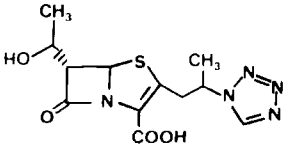

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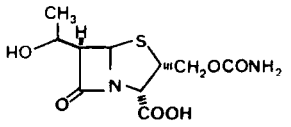

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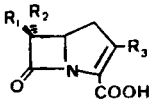
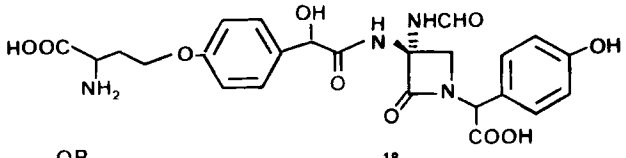


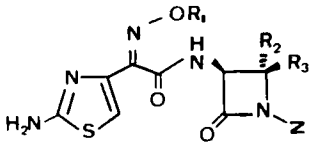
16 $R_1 = H$ $R_2 = CH_3OCH_2CH(OH)$ $R_3 = -SCH_2CH_2NH_2$

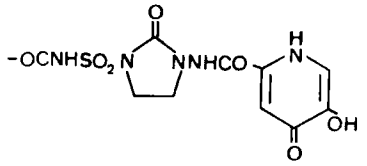
17 $R_1 = CH_3CH(OSO_3H)-$ $R_2 = -H$ $R_3 = -SO_2H$

Monocyclic ß-Lactams - A new class of monocyclic ß-lactams containing a 3α-formamido group, exemplified by formacidin C(18), was isolated from a bacterium of the genus Flexibacter.[107,108] Like their cephalosporin relatives the chitinovorins, described in 1984, formacidin C was highly stable to ß-lactamase. However, 18, though potent, has a very narrow spectrum of activity, being limited to Pseudomonas, Proteus and Alcaligenes. SAR studies were published [109] which led to the selection of carumonam (AMA-1080, 19) as a candidate for extensive biological[110-112] and subsequent clinical evaluation. The results of SAR studies on 1-oxysulfonic acid derivatives of the monobactams (monosulfactams) have been reported.[113,114] The antibacterial activity of these derivatives is, in general, limited to gram-negative organisms. Interestingly, their oral absorption properties appear to be structurally specific to the C-4 dimethyl compounds suggesting that absorption occurs by a facilitated transport system. SQ30,213 (20) was selected for further biological evaluation.[115,116] A 1-sulfonylaminocarbonyl derivative, SQ83,360(21), was selected for further study following the synthesis and testing of numerous analogs.[117] This compound exhibited exceptional activity versus P. aeruginosa.[118,119] SAR studies also were carried out on 1-oxyacetic acid derivatives.[120,121] Again, bioactivity was limited to gram-negative pathogens. Publications also appeared on the effects of having heteroatom substitution at the 4-position of the monocyclic ring,[122] the discovery of the monobactams[123] and aztreonam[124] and on a naturally-occurring chlorinated nocardicin.[125,126]



18



	R_1	R_2	R_3	Z
19	$-CH_2COOH$	$-H$	$-CH_2OCONH_2$	$-SO_3H$
20	$-CH_2COOH$	$-CH_3$	$-CH_3$	$-OSO_3H$
21	$-C(CH_3)_2COOH$	$-H$	$-H$	

β-Lactamase Inhibitors - Further studies with the penicillanic acid sulfone derivative, YTR-830, demonstrated that when combined with ampicillin it is as effective as clavulanic acid and more effective than sulbactam in inhibiting β-lactamases, especially those of chromosomal origin.[127] A review of the mechanism of action of B-lactamase inhibitors was published.[128] Three reports also appeared describing both mechanistic and biochemical aspects of β-lactamase inhibition.[129-131]

Biosynthetic Pathways and Enzymes - A number of publications appeared in 1985 which describe various aspects of the biosynthesis of β-lactams. The stereochemistry of cephalosporin C biosynthesis starting from chiral methyl valine was reviewed.[132] One study showed that the β-methyl of penicillin N labeled from the chiral methyl of valine suffered complete epimerization in the oxidative ring expansion to deacetoxycephalosporin C.[133] The conversion of the latter to deacetylcephalosporin C in Acremonium strictum was shown to proceed with retention of configuration.[134] The incorporation into isopenicillin N of ^{13}C- and ^{15}N-labeled precursors using a cell-free system from C. acremonium was studied.[135] The enzymes deacetoxycephalosporin C synthetase and deacetoxycephalosporin C hydroxylase were shown to be distinct and separable enzymes in S. clavuligerus but were not separable in C. acremonium.[136] Two general reviews of β-lactam biosynthesis were published.[137,138] Biosynthetic studies also appeared on carbapenems,[139] thienamycin[140,141] and clavulanic acid.[142,143] Using purified biosynthetic enzymes from C. acremonium and S. clavuligerus sulfur-containing side-chain derivatives of penicillins and cephalosporins were prepared and found to be more active than their naturally-occurring counterparts.[144-146] Preliminary amino acid sequencing has been carried out on the active site of PBP 1b[147] and PBP 5[148] from E. coli and on the terminal amino acids of isopenicillin N synthetase from C. acremonium.[149] Active-site specific mutations in PBP 3 of E. coli produced an enzyme with reduced affinity for cephalexin.[150]

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Chapter 14. Quinolone Antibacterial Agents

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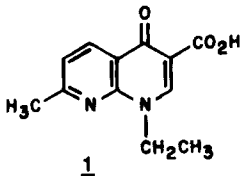
Introduction* - The renewed interest in quinolone antibacterials that began 5 years ago with the first descriptions of norfloxacin (NOR), pefloxacin (PEF), and enoxacin (ENO) has continued unabated. After two decades of only modest improvements in the antibacterial potency, the activity of the new fluoroquinolones far surpasses that of the original quinolone, nalidixic acid (NAL, 1).[1-6] The most significant changes made in the quinolone nucleus, addition of fluorine and piperazine, have provided these fluoroquinolones with activity against Gram-negative bacteria that rivals that of the major classes of antibiotics.[3,5]



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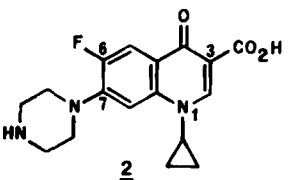

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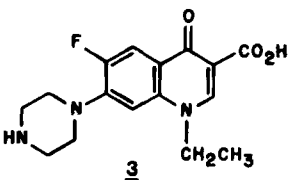


3

Previously relegated to the treatment of UTI, the increased potency of the new fluoroquinolones holds promise for a greatly expanded clinical use. This includes the potential for treatment of bacterial prostatitis, gastroenteritis, osteomyelitis, some pneumonias and infections caused by multiply-resistant Enterobacteriaceae and methicillin-resistant staphylococci.[1] The possibility for such clinical use has motivated at least eleven major pharmaceutical firms to engage in the R&D of quinolone antibacterials. This has resulted in clinical trials with at least eight different fluoroquinolones throughout the world, of which four have received approval for clinical use outside the U.S. The first of these new fluoroquinolones is expected to reach the U.S. market in 1986.

Relative to the first commercially introduced fluoroquinolone, NOR, subsequent analogues have shown greater oral absorption (PEF, ENO), increased serum half-life (PEF), overall increased potency in vitro (CIP) and an increased spectrum to include Gram-positive cocci (CI-934) and anaerobic bacteria (DIF).[1,3] Along with the intense effort to design more potent derivatives, a major thrust in development has been to provide formulations acceptable for intravenous use. If successful, fluoroquinolones could provide both oral and parenteral therapy for a broad range of bacterial infections in both hospitalized and outpatient populations.

The chronology of quinolone development in chemistry and biology prior to 1985 was the subject of a previous review.[3] This report will complement recent reviews describing the mechanism of action and resistance,[5,6] spectrum,[4,5] toxicity,[1] pharmacology[1] and SAR's[7,8] of the quinolone antibacterials and focus on the relevant literature of 1985. The biochemical target of the quinolones is the bacterial DNA gyrase, an example of the type II topoisomerase class of enzymes

*Abbreviations used in this report: AMI-amifloxacin; CIP-ciprofloxacin; DIF-difloxacin; ENO-enoxacin; NAL-nalidixic acid; NOR-norfloxacin; OFL-ofloxacin; PEF-pefloxacin; UTI-urinary tract infections.

that are described in a separate report in this Volume.[8a] For those quinolones that have been studied clinically, human pharmacokinetics and efficacy will be stressed. Where there has been little or no human data reported, preclinical laboratory data will be presented. Whereas NOR was the dominant quinolone during 1980-84, CIP became the in vitro class standard during 1985 with OFL rapidly emerging as the market standard.

Ciprofloxacin (2) is the most potent of the quinolone antibacterials.[4] As with other quinolones, its in vitro activity is not enhanced, nor antagonized by the presence of other antibacterial agents[9,10] but it is reduced by divalent Mg.[11]

The pharmacokinetics of CIP have been determined following oral and intravenous administration of single or multiple doses.[12] Peak serum concentrations were proportional to the dose with values of 0.38 or 3.62 μg/ml achieved after single oral doses of 100 or 1000 mg.[13] No significant differences between HPLC and microbiological assay values were found in the serum of singly[14] or multiply[15] dosed subjects. Significant differences were noted, however, by the two methods in two other studies.[12,14] The level of CIP metabolites in urine, as percent of total drug in urine, was greater for a 50 mg dose administered orally (42.7%) relative to the same dose given iv (29.7%).[16] These data suggest a first pass metabolism effect of CIP[16] leading to biologically active metabolites in the bile[14] and in the urine.[12,14,16,17]

Most studies show similar pharmacokinetic profiles for CIP with oral and iv dosing.[12-14,16,18,19] When compared in the same study, CIP showed approximately 60 to 77% bioavailability by the oral route.[16,17] CIP is widely distributed giving volumes of distribution values ranging from 2.0 to 2.7 l/kg after iv doses of 50 to 200 mg.[16-18,20] Since normal body water is 0.6 l/kg, this indicates distribution into secondary tissue compartments. CIP is reportedly taken up into human neutrophils where it exerts a bactericidal effect against intracellular staphylococci.[21]

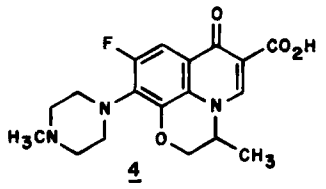
CIP has been used clinically to treat a variety of bacterial infections including bacteremia,[22] deep soft tissue infections[22-24] and abscesses,[23] GI,[22,25,26] UTI,[22-24,27,28] and pulmonary[22-24] infections, chronic otitis,[23] osteomyelitis,[22-24] gonorrhea[29] and GI decontamination.[26] Instances have been reported of CIP-resistant bacteria from patients receiving oral or iv therapy[30,31] and one report of hematuria[32] has appeared. In one study side effects were noted in 12 of 42 patients with liver enzyme elevations being the most frequent (5 patients).[23]

Norfloxacin (3) was the first of the 6-fluoro-7-piperazinylquinolones to reach the market (1983) and should be available for clinical use in the U.S. in 1986. An excellent review of the antibacterial, pharmacokinetic and therapeutic properties of NOR was recently published.[33] The in vitro potency of NOR is less than that of CIP or OFL and the oral absorption is less than that of OFL or ENO.[3] Nonetheless, NOR is active against those bacterial pathogens responsible for infections of the eye,[34] the urinary tract,[35,36] gastroenteritis,[37,38] and some genital pathogens,[39] while less active against anaerobic bacteria[40] and some selected bacteria such as Pseudomonas maltophilia and S. faecalis.[41]

The normally prescribed dosage of NOR (400 mg, po) provides serum levels near 1.5 μg/ml. With 30% of the dose excreted into the urine (peak concentrations of 180 to 480 μg/ml),[33] NOR has proven to be effective therapy for both uncomplicated and acute urinary tract infections.[33,42-44] There is little excretion of NOR into the bile, but NOR has been measured in several other tissues and body fluids,[33] including human prostatic tissue[45] and prostatitis has been effectively treated with NOR.[46] The drug reduces the Enterobacteriaceae in the anal and vaginal flora.[44] NOR may thus be effective in prophylaxis against bacterial sepsis

in neutropenic patients.[33] Other therapeutic uses for NOR may include ear, nose and throat infections and some infections of the respiratory tract other than those cause by pneumoncocci. Clinical trials for these indications have been conducted in Japan.[33]

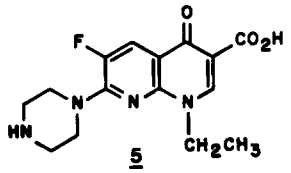
Ofloxacin (4) potency is close to that of CIP[4,38,39,47,48] and greater than CIP for some bacteria.[39,47] This tricyclic quinolone has shown excellent potency in vitro against enteropathogens,[38] mycobacteria,[48] anaerobes[49] and genital pathogens.[39] The drug appears to penetrate cellular membranes and inhibits bacterial growth within host tissues.[47,51] The protective action of OFL in experimental infections indicates that the drug is well absorbed and this observation has carried over into clinical studies.[50-52]



4

OFL has been compared with trimethoprim/sulfa, nitrofurantoin, doxycycline and cefuroxime in clinical trials in Europe and Japan. Single dose (100 mg, po) therapy has been successful for UTI[53] while longer treatment times (7-10 days) and higher doses have been used more frequently.[54] More attention has been focused recently on respiratory infections[54] where 200 mg BID has proven more effective than doxycycline[55] or cefuroxime.[53,55] Other bacterial infections successfully treated have included biliary,[56] pyelonephritis, prostatis, osteoarthritis, traumatic and post-operative wound infections.[54] Gynecologic infections have responded to OFL with cure rates (95%) equal to minocycline against Chlamydia[55] and long term treatment (6-8 months) has been successful for treating otherwise resistant cases of tuberculosis.[57] Pharmacokinetic measurements of OFL in lung[58] and prostatic[59] tissues, as well as bile,[53] are consistent with observed clinical efficacy. The few side effects reported[55] include nervousness, GI upset and pain, skin reaction and transiently elevated liver transaminases without any signs of renal toxicity.[60] OFL levels in serum (ca. 4 μg/ml) and blister fluid (ca. 2 μg/ml) have been measured following oral dosing while bile contained 26% of the OFL as glucuronide conjugates.[53] The rate of oral drug absorption and the serum half-life (9.0 hr) was reduced by food[59] while in uremic patients the plasma half-life increased from 4.4 hr to 13.2 hr with a reduction in urine recovery from 74% to 20% relative to patients with normal glomerular filtration.[59] One crossover comparative pharmacokinetic study showed oral OFL to have 2-fold greater serum levels and urine recovery relative to the same oral dosage (200 mg) of CIP.[61] The synthesis and antibacterial activities of both OFL enantiomers have been reported.[62] The levorotatory isomer was 8- to 128-fold more active in vitro than the dextrorotatory isomer against a variety of Gram-negative and Gram-positive bacteria.

Enoxacin (5) is generally less potent than CIP or OFL and particularly so with respect to streptococci.[4,63,64] However, ENO is more potent in vitro against Gram-negative bacteria including Pseudomonas than aztreonam or the aminoglycosides.[65] ENO is well absorbed orally[66,67] reaching steady state serum concentrations (peak and trough values of 3.5 and 1.3 μg/ml, respectively) by the third day of dosing (400 mg, BID, 7 days).[68] Serum half-life values range from 4.4 to 6.7 hr[67,68] with 40% to 70% of the dose appearing in the urine[66] as ENO and 4 active metabolites.[68] One report described single intravenous doses (10 to 800 mg) to human volunteers as being well tolerated and providing slightly higher plasma levels (5.8 μg/ml) than the same oral dose of 800 mg (4.0 μg/ml).[67] ENO is capable of penetrating human leukocytes and equally potent as rifampin in killing ingested



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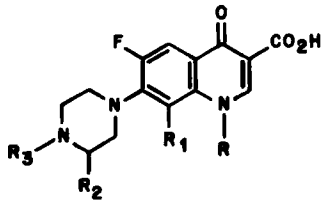
bacteria.[69] The capability of tissue penetration is also seen in pharmacokinetic studies where levels of ENO in human sputum, prostatic tissue and bile exceeded serum or plasma levels.[67] ENO was also found in human tears, saliva, blister fluid and middle ear exudates.[68] Similar results have been found for ENO penetration of prostatic tissue and the CSF of dogs.[68,70] This ability to cross the blood-brain barrier may contribute to the CNS-related side effects observed in clinical trials.[68,71-74] Clinical trials to date have relied upon oral ENO therapy (generally 200 to 400 mg/day, BID) to treat infections of the respiratory,[75,76] urinary,[72,74] GI and biliary tracts,[68] skin and soft tissue,[68] as well as gynecological[76] and ophthalmological infections.[68] The majority of these trials with respiratory or urinary tract infections have shown ENO therapy to be 70 to 80% effective[68] and in the case of acute gonorrhea, 100% effective.[76] Side effects in humans were reportedly dose related[68] increasing from 0.9% with 200 mg/day to 13.3% with 900 mg/day oral doses and may have been related to an interference with theophylline metabolism in some cases.[68,75]

Pefloxacin (6) and NOR were the first of several 6-fluoro-7-piperazinylquinolones to be described. PEF has in vitro activity similar to NOR and ENO against Enterobacteriaceae and P. aeruginosa but is 4- to 8-fold more potent against S. aureus.[77] CIP is considerably more potent than PEF against Gram-negative organisms but they generally show similar activity against Gram-positive cocci. In experimentally induced Pseudomonas pneumonia in guinea pigs, PEF and CIP at the same dose (im) were highly efficacious in increasing survival times and decreasing bacterial counts in the lungs of surviving animals.[78] Against an experimental infection model with methicillin-resistant S. aureus, PEF at 30 mg/kg, (im) was as efficacious as vancomycin (25 mg/kg, iv) while cephalothin was ineffective.[79]

In humans, PEF exhibited a relatively long serum half-life.[1] At a dose of 400 mg (po or iv), a serum half-life of 11 hr was observed.[80] Areas under the plasma concentration curves were essentially identical indicating complete oral bioavailability. At this same dose, peak serum levels of PEF were 3.8 and 4.2 μg/ml for po and iv administration, respectively.[1] Urinary recoveries of PEF were 11% of a 400 mg oral dose providing concentrations well in excess of the MIC's against most uropathogens.[80] PEF is extensively metabolized with NOR being the major metabolite.[1] Biliary excretion of PEF, either as intact drug or metabolites, can account for 20-30% of an oral dose and its pharmacokinetics were studied in patients with cirrhosis of the liver.[81] Following a single dose of PEF (8 mg/kg, iv) the mean serum half-life, although highly variable, was significantly longer (35 hr) in cirrhotic patients than in normal subjects.[81]



6, R = CH_2CH_3, R_1 = H, R_2 = H, R_3 = CH_3
7, R = $NHCH_3$, R_1 = H, R_2 = H, R_3 = CH_3
8, R = 4-FC_6H_4, R_1 = H, R_2 = H, R_3 = CH_3
9, R = 4-FC_6H_4, R_1 = H, R_2 = H, R_3 = H
19, R = CH_2CH_2F, R_1 = F, R_2 = H, R_3 = CH_3
20, R = CH_2CH_3, R_1 = F, R_2 = CH_3, R_3 = H

Amifloxacin (7) is the only 6-fluoro-7-piperazinylquinolone reported that does not have a carbon directly attached to the 1-position; it has instead a methylamino appendage.[82] AMI has broad in vitro activity comparable to NOR, PEF and ENO but is less active than CIP.[4,82,83] Like other quinolones AMI has relatively low activity against most anaerobes. It was more effective in vitro than aztreonam in inhibiting amikacin-resistant P. aeruginosa.[84] Against Gram-negative clinical isolates from cancer patients, AMI, HR 810, and imipenem exhibited potent in vitro activity which was considerably greater than that exhibited by ceftazidime.[85] AMI and CIP had similar in vitro activity against 29 Legionella isolates but both were much less potent than rifampin.[86]

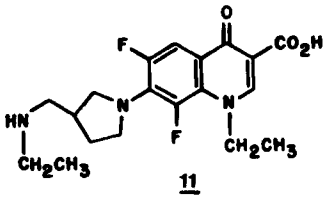
Pharmacokinetic studies with rats indicated that AMI, as with many quinolones, was well absorbed orally and rapidly excreted into the urine.[82,87] The elimination half-life of AMI was 2.2 hr in rhesus monkeys given 10 mg/kg (base equivalents) of AMI mesylate intravenously.[88] Concentrations of AMI in prostatic secretion and prostatic interstial fluid of dogs medicated with AMI (iv) exceeded the MIC's of most pathogens involved in chronic bacterial prostatitis.[89]

Difloxacin (A-56619, 8) and A-56620 (9) are 1-(4-fluorophenyl)-substituted quinolones which exhibit excellent in vitro and in vivo activity against a wide variety of organisms, including anaerobes.[90,91] Against E. coli and E. cloacae, A-56620 showed comparable in vitro activity to CIP ($MIC_{90} \leq 0.06$ μg/ml) but was generally less active than CIP against other Gram-negatives.[92] A-56620 was less active than CIP but two to eight times more active than NOR against Gram-positive isolates. Against Chlamydia trachomatis DIF exhibited similar in vitro activity as tetracycline while A-56620 and OFL were somewhat less potent.[93] DIF, A-56620, and CIP had similar in vitro activity against Bacteroides (including B. fragilis), Clostridium and other anaerobe spp. while NOR was considerably less active,[94-96] although clindamycin was more potent than any quinolone tested.[96] In rats and dogs DIF showed essentially complete oral bioavailability resulting in high peak serum levels and extended serum half lives relative to other quinolones.[90,91]

In addition to 1-piperazinyl and 4-methyl-1-piperazinyl at position-7, the 3-amino-1-pyrrolidinyl group was also shown to impart good activity to these aryl fluoroquinolones.[97] The in vitro activity of one such analogue, A-60969 (10) was 2- to 16-fold more potent than CIP against Gram-positive organisms and approximately 10-fold more potent than CIP against B. fragilis. Against P. aeruginosa and enteric bacteria, A-60969 was approx. 4-fold less potent than CIP.[98] Qualitatively similar comparative data were noted in mouse protection tests against Gram-positive and Gram-negative organisms.[99]

Both enantiomers of an aryl fluoroquinolone have been prepared.[100] They differ from DIF only at the 7-position; instead of a piperazine moiety these new agents have a 2-hydroxymethyl-1-pyrrolidinyl substitution. The (R)-enantiomer had approximately 60- and 30-fold greater in vitro activity against E. coli and S. aureus, respectively, than the (S)-enantiomer. Pirfloxacin, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1H-pyrrol-1-yl)-3-quinolinecarboxylic acid, has been synthesized and has in vitro activity reported to be similar to ENO.[101]

CI-934 (11) has broad in vitro activity and is particularly effective against Gram-positive bacteria.[102] For example, CI-934 is 4-fold more potent in vitro than OFL against staphylococci. Against Pseudomonas spp., CI-934 has much higher MIC's than against the Enterobacteriaceae. The in vitro activity of CI-934 against B.



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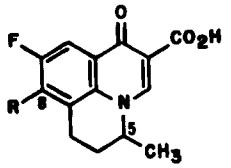
fragilis was comparable to CIP but 2-fold and 8-fold less potent than clindamycin and metronidazole, respectively.[103] CI-934 was 4-fold more potent than CIP and OFL against Clostridium difficile.[104] Oral efficacy of CI-934 has been demonstrated in mice experimentally infected with E. coli, S. aureus, and S. pyogenes.[105] Upon oral administration of the agent (25 mg/kg) to dogs a peak plasma level of 5.8 μg/ml occurred at 3 hr with an apparent half-life of 8.4 hr.[106] CI-934 had an oral bioavailability of 57% in dogs.

S-25930 (12) and S-25932 (13) are two new analogues of flumequine (**14**)[107] in which the hydrogen at position-8 has been replaced by methyl and 1-imidazolyl, respectively. Against a variety of Gram-negative and Gram-positive organisms, including anaerobes, S-25930 and S-25932 were 2- to 4-fold less potent in vitro than CIP and had activity similar to ENO and OFL.[108] In another in vitro study S-25930 and S-25932 displayed activity similar to CIP against streptococci, Neisseria spp., H. influenzae, Bacteroides spp., Clostridium spp., and anaerobic Gram-positive cocci.[109] Against the Enterobacteriaceae and P. aeruginosa the two quinolones were 20-fold and 60-fold less active than CIP, respectively. S-25930 was approximately 2-fold more active in vitro than S-25932. S-25930 showed excellent activity, while S-25932 exhibited poor in vitro activity, against an isolate of N. gonorrhoeae resistant to rosoxacin but sensitive to NAL.[110] Based on this and studies with other N. gonorrhoeae isolates, quinolone resistant Neisseria were divided into two groups. One that was susceptible to quinolones having heterocyclic (e.g. 1-piperazinyl, 4-pyridinyl, or 1-imidazolyl) substitution; the other susceptible to quinolones having methyl or hydrogen substituents (e.g. S-25930, NAL, or flumequine). In vivo comparison of S-25930 and NOR in mouse protection tests (po) showed a similar potency/spectrum profile as the in vitro experiments.[111] In the adult rat, S-25930 was consistently detected in brain tissue while NOR was not.[112] Comparison of the in vitro properties of both optical isomers of S-25930 indicated the (S)-enantiomer to be more potent than the (R)-isomer.[113]



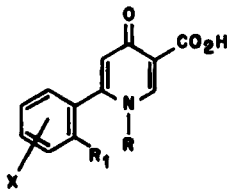
12, R = CH_3

13, R = 

14, R = H

Other New Quinolones

A series of novel mono- (**15**) and tricyclic (**16**) analogues of the quinolones has been reported.[114] Ro 13-5478 (**15**; X=4-NMe_2) was the most potent member of that subseries having in vitro activity against E. coli and S. aureus comparable to NAL. Because several of the monocyclic compounds showed CNS stimulation, an attempt was made to reduce the CNS activity by restricting the conformation of the phenyl ring with respect to the 4-pyridone ring. To that end a sterically encumbered variant **15** (X,R_1=2,6-diMe-4-NMe_2) was made in which the rings are nearly perpendicular. This analogue lacked CNS stimulation, however, it was devoid of antibacterial activity. Several examples of tricyc-



15, R = CH_2CH_3, R_1 = H

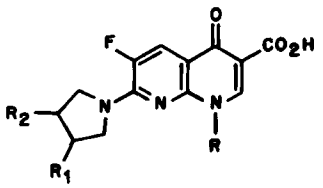
16, R_1,R = CH_2CHCH_3

lic derivatives **16**, in which the rings are planar, were found to have in vitro activity against E. coli and S. aureus. The most active tricyclic was Ro 14-9578 (**16**; X=3',4'-OCH_2CH_2O-) and had antibacterial potency greater than NAL. In contrast to the chirality studies of S-25930[113] the (R)-enantiomer of **16** (X=H) was considerably more potent in vitro than the (S)-isomer. Ro 13-5478 and Ro 14-9578 had similar in vitro DNA gyrase (E. coli) inhibitory activity that was somewhat greater than NAL. Both compounds had substantially reduced activity against a NAL resistant E. coli. These data indicate that these mono- and tricyclic analogues act like the quinolones by inhibiting the A subunit of bacterial DNA gyrase.

A new series of 1-cyclopropyl-1,8-naphthyridines containing a 4-substituted-3-aminomethyl-1-pyrrolidinyl appendage at the 7-position has been reported.[115] One of the most potent analogs, AT-3765 (**17**), had in vitro and in vivo (mouse, iv) Gram-positive activity equal to or up to 10-fold greater than CIP.[116] In similar tests against E. coli and P. aeruginosa, however, CIP was 2-fold more active than AT-3765. The trans-isomer of AT-3765 showed comparable in vitro activity as did the fluoro (cis and trans) and deschloro analogues. A related quinolone, AT-3295 (**18**), was shown to have similar in vitro activity to CIP against Gram-negative bacilli (including P. aeruginosa) and anaerobic bacteria.[117] However against Gram-positive cocci, AT-3295 was 3-fold more potent than CIP. Upon comparison of AT-3295 and CIP in mouse protection models (po and iv), a similar profile to the in vitro study emerged.[118] The two diasteriomers of AT-3295 showed comparable in vitro activity.

AM-833 (**19**), although somewhat less active than CIP in vitro, was 2- to 8-fold more active than CIP when administered orally to mice infected with S. aureus, P. aeruginosa, K. pneumoniae, or S. marcescens.[119] Following a single oral dose of 10 mg/kg of AM-833 or CIP to mice, mean peak serum levels were 2.4 to 0.4 μg/ml, respectively.[120] Lung and kidney levels of AM-833 were much higher than those of CIP.

Another 6,8-difluoroquinolone, NY-198 (**20**), has a 3-methyl-1-piperazinyl appendage at position-7 instead of the more common 4-H- or 4-methyl-1-piperazinyl moiety.[121] It has broad in vitro activity comparable to NOR and OFL and is up to 4-fold more potent in vivo. Following a 20 mg/kg oral dose of [^{14}C] NY-198 to dogs, a Cmax of 11.1 μg/ml (total radioactivity) was observed within 1 hr with a serum half-life of 6.9 hr. NY-198 showed complete oral absorption in dogs with 61% of dose excreted into the 0-48 hr urine.[122]



10, R = 2,4-diFC_6H_3, R_1 = H, R_2 = NH_2

17, R = c-C_3H_5, R_1 = Cl, R_2 = CH_2NH_2 (cis)

18, R = c-C_3H_5, R_1 = CH_3, R_2 = NH_2

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CHAPTER 15. DEVELOPMENTS IN MICROBIAL PRODUCTS SCREENING

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INTRODUCTION - Over the course of the past three decades the face of natural products discovery from microbial sources has undergone dramatic metamorphosis from blind antibiotic screening, to target-directed assays for antimicrobials, through a variety of specific enyzme inhibitor screens, antigen-antibody screens and now to the use of receptor-ligand binding assays. Strategies used in the discovery of drugs from natural sources have been reviewed previously in this series.[1] Decreasing numbers of clinically useful drugs following major systematic searches of plant and microbial sources has led to discouragement, on the part of some, in the prospects for natural products research as a fruitful approach to drug discovery.[2,3] Others take the view that natural products research, in general, and fermentation, in particular, may represent an avenue for renewed industrial productivity.[4] Traditionally, microbial products discovery has focused on antimicrobial and anti-tumor agents. Compounds with significant pharmacological activities have not typically been discovered, and the conventional reason cited for this is the difficulty of adapting testing methodology to fermentation broths.[4] With the recent explosion in molecular biology, the development of new assay procedures coupled with the application of facile screening methods appears to be favorably altering the discovery rate of microbial products with a range of pharmacological activities. Recent data suggests that while there continues to be an annual discovery rate of approximately 50 new antimicrobials, there has also been a notable increase in the rate of discovery of new pharmacologically active agents, ranging from a handful in 1981/1982 to 17 in 1985 (Table I). In contrast, there have been few reports describing immunoregulatory molecules or agrochemicals from microbial sources. Comprehensive reviews of antimicrobial,[5,6,7,8,9] pharmacological,[10,11] immunoregulatory,[12] antitumor[13,14] and general approaches[15,16,17,18] to screening programs have appeared over the past decade. In this review, we will describe signficant new discoveries from microbial sources which have been reported during the past few years along with novel screening assays and methodologies. It will not cover antitumor antibiotics.

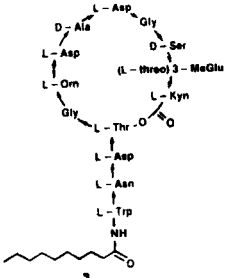
ANTIBACTERIALS - The development of targeted screening systems has had a profound effect on microbial products discovery. ß-Lactam screens exemplify the trend toward screens aimed at specific metabolic targets. The first ß-lactams were detected by agar diffusion of antimicrobial activity from colonies of microbes through agar containing whole cells of susceptible gram positive bacteria. Over the years, these have been successively replaced by the detection of morphological changes,[19] supersensitive bacteria,[20] ß-lactamase inhibition[21] and D, D- carboxypeptidase inhibition.[22] Recently, novel approaches employing ß-lactamase induction,[23] and a chromogenic

substrate[24] for detecting D, D-carboxypeptidase inhibitors were described, along with the discoveries of a number of monocylic ß-lactams[25-30] and carbapenems.[31-33] The common features of ß-lactam screens that have lead to their success are specificity and sensitivity. This was elegantly exemplified in reports on the development and application of monoclonal antibodies to an enyzme-linked immunosorbent assay for the detection of cephalosporins.[34,35] This system is sensitive (detection limit below 1 µg/ml) and specific for acetyl or acetoxy cephalosporins. Indeed this was a first generation monoclonal antibody detector that was probably overly compound specific, but provided an important assessment of the hypothesis on the basis of which class-specific antibodies could be developed.

Other antibacterials have recently been sought that are effective against the emerging ß-lactam resistant gram positive strains. This has led to the development of semi-synthetic lipopeptide antibiotics (1)[36] and a series of glycopeptide antibiotics. Reports of glycopeptides of the vancomycin class include aridicins (2),[37] A41020,[38] actaplanin,[39] A16686[40] and N-demethylvancomycin.[41]

TABLE 1
REPORTS OF NEW MICROBIAL PRODUCTS IN J. ANTIBIOTICS 1981-1985

BIOLOGICAL ACTIVITY	1981	1982	1983	1984	1985
Antimicrobial	34	41	54	43	41
Antitumor	20	15	13	8	22
Pharmacological	5	3	9	18	17
Immunoregulatory	0	1	0	0	0
Agrochemical	1	0	1	2	0



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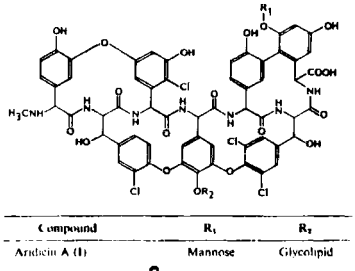

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The discovery of new glycopeptide antibiotics was markedly enhanced by the development and application of innovative screens. In a holistic approach, the strain selection, fermentation and specificity of the screen were shown to have a profound effect on the detection rate.[8] Novel antibacterial agents of the vancomycin class were detected at a rate of 1 per 320 cultures tested. The effectiveness of this approach was in large part due to the use of a mechanism-based screen employing the bacterial cell wall receptor to which vancomycin class antibiotics bind. The successful application of polyvalvent vancomycin antibodies to screening for new glycopeptides (Fig. 1) was reported.[42] This technique was previously described for aminoglycoside screens.[43] These assays are highly sensitive, specific and amenable to automation for high throughput. The

heterogenous microplate enzyme immunoassay for aminoglycosides had a sensitivity of 10 pg/ml for gentamicin and cross-reacted with a large number of aminoglycosides with the exception of neomycin. Furthermore, the assay was insensitive to other antibiotics and detected strains producing sub-antibacterial concentrations of aminoglycosides.

FIG 1

COMPETITIVE VANCOMYCIN ELISA PROCEDURE

Antibody Coating (Microtiter Wells)

↓

Blocking Step (PBS-BSA)

↓

Add Sample/Broth

↓

Add Vancomycin-Enzyme Conjugate (Alkaline Phosphatase)

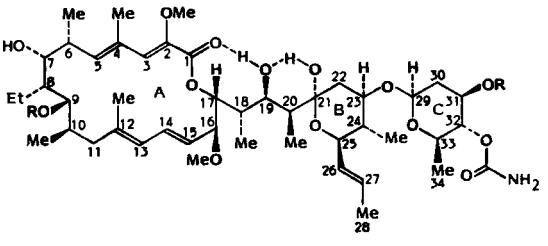
↓

Add Substrate (p-Nitrophenylphosphate)

↓

O.D. Reading (Microplate Reader)

The incidence of new antibiotics isolated from actinomycetes, fungi and eubacteria does not indicate that any particular group has a higher propensity to produce new compounds.[18] Thus, the high incidence of fungal derived products described from the U.K. is reported to be an indication of the intensity of screening these products whereas the concentration on actinomycetes in Japan has similarly influenced the predominance of this source of antibiotics. Also, the use of rarer species does not always increase the discovery rate since, for example, during 1974-1980 the number of Actinoplanes derived antibiotics increased by 34 while those from Streptomycetes increased by 847 new compounds. There are exceptions and the discovery of the acylated glycopeptides of the aridicin group was due to the isolation of a new genus of actinomycetes designated Kibdellosporangium.[37]



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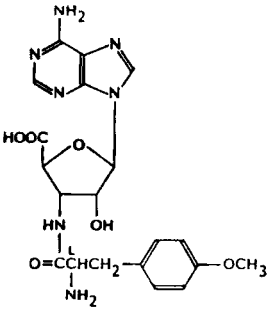

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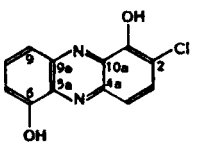

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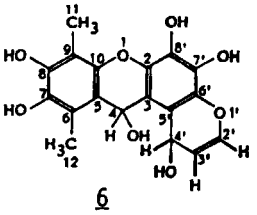

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ANTIFUNGALS - Antifungal chemotherapy was last reviewed in these reports in 1984.[44] Whereas that review concentrated mainly on the synthetic azole antifungal drugs, this section covers new antifungal agents of various structural classes which have been reported from microbial sources during the last three years. The macrolides emerge as the major class of novel compounds reported during the period (Table 2), accounting for a quarter of the new agents. The structural variety of antifungals that have been isolated from microbial sources are illustrated by concanamycin A(3), chryscandin (4), chaetiacandin (5) and mycoversilin (6).

TABLE 2

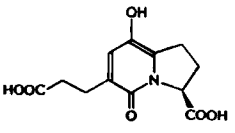
ANTIFUNGAL AGENTS REPORTED IN J. ANTIBIOTICS 1983-1985
(numbers in brackets indicate total compounds reported)

ANTIBIOTIC TYPE	REFERENCE	ANTIBIOTIC TYPE	REFERENCE
Polyene (6)	45	Nitrosohydroxylamine (1)	54
Macrolide (13)	46	Pyrrole (4)	55
Lactone (4)	47	Phenazine (1)	56
Basic (4)	48	Anthraquinone (1)	57
Peptide (2)	49	Dibenzopyran (1)	58
Nucleoside (3)	50	Phosphonamide (1)	59
ß-Methoxyacrylate (2)	51	Indolocarbazole (1)	60
Quinone (5)	52	Azetidine (1)	61
Ansamycin (1)	53	Papulacandin (1) (glycolipid)	62,63
		Acyltetramic acid (1)	64

Screens for antifungal agents include the preferential inhibition of fungal growth (mycoversilin[58]), cell elongation of the fission yeast Schizosaccharomyces (leptomycins[47]), inhibition of imperfect fungi (histidinomycin[48]), potentiation of polyenes (neo-enactin[64]), and inhibition of cell-wall synthesis (phosphazomycin A).[59] However, the search for new and safe antifungal antibiotics is limited by the specificity and sensitivity of the screens employed. Improved screens are limited by the paucity of information on the differential metabolism of fungal and mammalian cells and the lack of good genetic and cloning systems for clinically important fungi such as Candida albicans. This is well recognized and progress is being made toward overcoming current limitations in screen development by cloning of C. albicans genes into Saccharomyces cerevisiae.[65,66] Thus, it is technically feasible to complement a strain of S. cerevisiae defective in a crucial enyzme or structural protein with genes cloned from mammalian or C. albicans cells. By comparing the inhibition obtained with microbial broths against isogenic S. cerevisiae containing different eukaryotic genes it should be possible to screen for antibiotics that are selectively toxic for the C. albicans gene

product. A further limitation in screening directly against C. albicans is its generally low sensitivity to antibiotics, due in part to permeability problems, that could be improved by cloning complementary genes into S. cerevisiae or other fungi.[66] Compounds having the desired specificity could subsequently be derivatized to improve potency.

PHARMACOLOGICAL AGENTS - The contribution of natural products to providing divergent chemical structures having affinity for the same protein is exemplified by the variety of inhibitors of angiotensin converting enzyme (ACE), Table 3. The facility with which specific screens for ACE inhibitors can be established has been elegantly illustrated.[67] This screen is comprised of a simple agar diffusion assay in which putative inhibitors are preincubated with the enzyme gel prior to being overlayed with a gel containing a chromogenic substrate. Originally, teprotide (SQ 20,881)[68] an isolate from the venom of Bothrops jararaca provided the impetus for synthetic proline-containing inhibitors of ACE that led to the development of captopril.[69] More recently, A58365A (7), a bicyclic conformationally-restricted analog of the synthetic ACE inhibitor, 2-methylglutaryl-L-proline was reported from Streptomyces chromofuscus.[70] One other non-peptidyl inhibitor, phenazine,[71] has been described as has a series of unusual peptide analogs including foroxymithine (8),[72] 15B2,[73] marasmine (9),[74] muraceins (10)[75] and ancovenin.[76] Aspergillomarasmine,[77] a fungal product, was shown to be a potent ACE inhibitor having an IC_{50} of 1uM.



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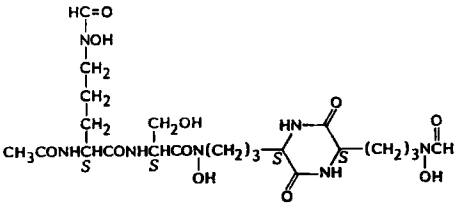

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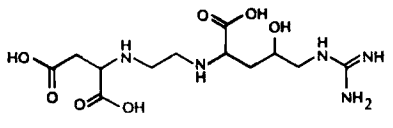

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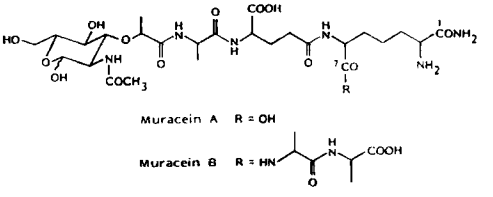

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A second pharmacological target screen for inhibitors of hydroxymethyl glutaryl-Co A reductase, has resulted in the discovery of novel molecules that would not have been designed using a rational synthetic chemistry strategy. These include the monacolins[78,79] (Table 3) and mevinolin, which is under development as a antihypercholesterolemic agent.[80]

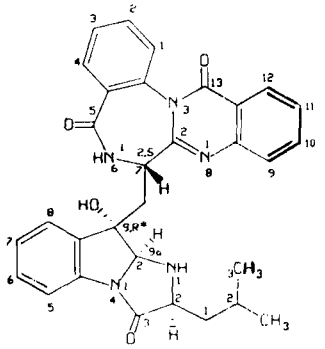
TABLE 3

MICROBIAL PRODUCTS HAVING PHARMACOLOGICAL ACTIVITIES (1983-1985)

Mechanism of Action	Microbial Metabolite
• Anti-hypertensive	
- Angiotensin converting enzyme inhibitor	A58365 A&B Foroxymithine, Muracein, L-681,176, 15B2, Phenacein, Ancovenin, Aspergillomarasmine
- Vasodilator	Amauromine
• Antihypercholesterolemic	Monacolins J, L & X, Dihydromonacolin X
• Antithrombotic	
- Platelet aggregation inhibitor	WF-5239, WS-30581 A & B
- Fibrinolytic	WB-3559 A, B, C, & D
• 5'-Nucelotidase inhibitor	Nucleoticidin, Melanocidin A & B
• Peptidase inhibitor	
- Caboxypeptidase	
A	S-α-Benzylmalic acid
B	Histargin
- Aminopeptidase	
leucine aminopeptidase	Bu-2743E
aminopeptidase B	Orphamenines A & B
dipeptidyl aminopeptidase III	Acetyl-L-leucyl-L-argininal
dipeptidyl aminopeptidase IV	Diprotins A & B
• Glycosidase inhibitor	Nojirimycin B D-Mannoic-ß-lactam Valiolamine
• Glucosyl transferase inhibitor	Trestatins Mutastein
• Phospholipase inhibitor	
- Phospholipase A_2	Plastatin, Luteosporin
- Phospholipase C	(S,S)-N,N^1-Ethylene diaminosuccinic acid
• Na^+, K^+ ATP'ase inhibitor	L-681,110
• Cyclic AMP-Phosphodiesterase inhibitor	Gyriseolic acid, Terferol, Acylpeptides
• Acetyl-CoA carboxylase inhibitor	Octyl pentanedioic acid
• Cholecystokinin antagonist	Asperlicin

A wide range of new pharmacologically-active microbial products have been reported in recent years (Table 3) including antithrombotics, and inhibitors of 5'-nucleotidase, carboxypeptidases, aminopeptidases, glycosidase, ATP'ase, cyclic AMP-phosphodiesterase, and acetyl-CoA carboxylase. The prolific rate of identification of these products is due to the harnessing of enzyme assays to provide specific, sensitive and high throughput screens.

The application of receptor-based screens to microbial products discovery has been predicted for some years.[12] During 1985, U.S. investigators reported the first example of a nonpeptide antagonist of a neuropeptide that has been isolated from microbial sources.[81] Asperlicin (11) is a new, competitve, cholecystokinin (CCK) antagonist that has 300-400 times the affinity for pancreatic, ileal and gallbladder CCK receptors than proglumide, a standard agent of this class.[81] In seeking to apply receptor binding assay techniques to the discovery of pharmacologically active substances from microbial sources, Japanese investigators also reported a novel ß-adrenergic receptor antagonist, MY-336a (12),[82] which was isolated from a Streptomyces fermentation.



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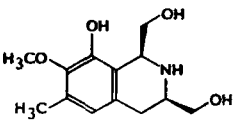

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CONCLUSIONS - The recent advances in screening technologies have demonstrated that the development of novel specific and sensitive screens is the key limitation to the exploitation of microbial products as further sources of pharmaceutics rather than the chemical variety and range of biological activities. Receptor based screens have demonstrated that molecular mechanism-based systems can be applied to microbial products and these may have further applications in immunotherapy and in novel approaches to cancer chemotherapy.

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Chapter 16. Oncological Aspects of Growth Factors

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A new arm of endocrinology has emerged within the last ten years as a result of the identification, purification, and molecular cloning of tissue growth factors.[1-3] Growth factors may be functionally defined as hormone-like proteins which can stimulate cell proliferation. In addition to controlling cell growth, these regulatory agents may also function as trophic or survival factors, chemoattractants, immune modulators, neurotransmitters, and as differentiation inducers or inhibitors.

The current interest in growth factors and their receptors and in the abnormal control of these cellular proteins has already had a major impact on the directions of basic and clinical research in such areas as endocrinology, immunology, neurobiology, developmental biology, and oncology. A large body of evidence has accumulated which suggests that these activities may be important in the etiology or progression of a variety of disease states.[3,4] Some of the most challenging and provocative results have come from defining the role(s) that growth factors or their receptors may perform in oncogenic transformation *in vitro* and possibly in tumor promotion and progression *in vivo*.[5-7] This review will focus on those more well-defined growth factors such as PDGF, EGF, TGFs, IGFs, and bombesin, which are associated with or synthesized by solid human tumor cells and their structural and/or functional relationship to specific oncogene and proto-oncogene proteins.

Growth Factors, Oncogenes, and Cell Growth - Tumorigenesis *in vivo* is a process consisting of several discrete steps (i.e., initiation, promotion, transformation, progression, and metastasis).[8] The conversion *in vivo* of a normal cell to a fully malignant, metastatic cell through each of these steps represents a process of continuous selection for a cell(s) which can readily adapt and proliferate in an environment with a minimum number of growth regulatory constraints. Changes in the responsiveness of cells to specific growth factors and in the interactions of cells with surrounding extracellular matrix components within the basement membrane or stroma can ultimately affect cell proliferation, motility, and differentiation and may be contributing factors to the processes of oncogenic transformation and subsequent tumor cell metastasis.[6,7,9-11] In this respect, we may consider cancer as a spectrum of different diseases which share a common characteristic of uncontrolled cell proliferation coupled in some instances to restricted or aberrant cell differentiation.[12] Transformation of mesenchymal or epithelial cells *in vitro* by oncogenic RNA (retroviruses), viruses, DNA tumor viruses, or by chemical carcinogens generally results in a partial or complete relaxation in the growth factor requirements of these cells for anchorage-dependent proliferation leading eventually to the acquisition of anchorage-independent growth (AIG).[6,7,13,14] This observation in conjunction with the recent knowledge that various retroviral oncogenes and their cellular homologs, the proto-oncogenes, code for specific regulatory proteins which control

discrete aspects of cell proliferation and/or differentiation has now led to a convergence in the study of growth factors and oncogenes.[6,15-17] The activation by point mutation or inappropriate overexpression of specific oncogenes or proto-oncogenes may confer growth factor autonomy upon cells.[7,17] This family of regulatory genes may code for proteins which are growth factors, which are portions of receptors for growth factors, or which are involved in the intracellular signal transduction pathway for growth factors.[6,15,17]

Platelet-Derived Growth Factor (PDGF) - PDGF is a heat stable, cationic (pI 9.8-10.2) polypeptide consisting of two peptide chains, A and B (Mr 12,000 to 18,000).[13,18] The native PDGF molecule (Mr 28,000 to 35,000) consists of either homo- or heterodimers of the A and B chains which differ in their degree of glycosylation and which are linked by interchain disulfide bonds.[18] PDGF is primarily but not exclusively synthesized in progenitor megakaryocytes and stored in the α granules of mature platelets. It is normally released into the blood following platelet degranulation during tissue injury and into serum following clotting. As such PDGF may be involved in the processes of wound repair and in plaque formation during atherosclerosis.[1,18] PDGF is a strong chemoattractant for smooth muscle cells and for stromal fibroblasts and may therefore be one of several growth factors along with endothelial mitogens and chemoattractants such as fibroblast growth factor (FGF) and endothelial cell growth factor (ECGF), which can contribute to the process of angiogenesis or neovascularization.[19,20] PDGF is also a potent mitogen for a variety of mesenchymal cells and for neural cell types but not for epithelial or endothelial cells.[1,3,18] The response of these target cells to PDGF is mediated through the binding of PDGF to specific high affinity cell surface receptors (Mr 170,000 to 180,000) which are associated intracytoplasmically with a tyrosine-specific protein kinase.[21]

The production of a PDGF-like activity(ies) has been demonstrated for fibroblasts and epithelial cells which have been transformed by a variety of retroviruses and DNA tumor viruses.[22,23] It has recently been demonstrated that this activity can compete with native PDGF for receptor binding and that the biological activity associated with this species can be effectively neutralized with anti-PDGF antibodies.[24,25] This suggests that PDGF and these cell-derived products are biologically, physiochemically, and immunologically related.[26] In 1983 the amino acid sequence of the B chain of PDGF and of the simian sarcoma virus (SSV) v-*sis* oncogene protein, $p28^{v\text{-}sis}$, was deduced. It quickly became apparent from these studies that these two proteins were virtually identical.[3,6,7,15-17] Cells which have been transformed by SSV express v-*sis*-related mRNA and synthesize and secrete a 20,000 to 24,000 Mr protein which is encoded by the viral mRNA and which is related to a homodimer of two B chains of PDGF.[18,24,26] The possibility that PDGF may function as an autocrine growth factor in this system stems from the observations that the differential susceptibility of various cells to transformation by SSV parallels their intrinsic responsiveness to exogenous PDGF and their ability to express PDGF receptors.[27] Moreover, following transformation, these cells lose their growth dependence upon exogenous PDGF and the number of cell surface receptors for PDGF is substantially reduced.[22,27] Both of these parameters correlate with an increase in tumorigenicity and possibly metastatic behavior *in vivo* and with the ability of these cells to synthesize endogenous PDGF-like peptides.[3,24,26,28] In addition, the c-*sis* proto-oncogene (chromosome 22 in humans) encodes a protein which is also identical to the B chain of PDGF.[3,6,18] If linked to a viral promoter, the c-*sis* gene is capable of transforming cells following transfection.[29] However, native,

exogenous PDGF by itself is incapable of eliciting all of the phenotypic changes which are observed following SSV transformation.[18,21] One possible explanation for this lack of complete mimicry by PDGF is that cells transformed by SSV and other unrelated retroviruses secrete not only a PDGF-like activity but also other growth factors such as transforming growth factors (TGFs), which may cooperate with this PDGF homolog in stimulating the growth of these cells through an autocrine mechanism.[5,7,22,30] This may be the case since anti-PDGF antibodies are either ineffective or only partially effective at relatively high concentrations in arresting the growth and in inhibiting foci formation of SSV transformed cells.[24,25] Alternatively, these results may suggest that this PDGF homolog is able to function as a mitogen intracellularly by its interaction with a PDGF receptor which is in transit to the cell surface and is therefore inaccessible to the action of these antibodies.[24]

Using complementary DNA (cDNA) probes for the c-*sis* gene, messenger RNA sequences related to c-*sis* have been detected in human glioma, *neuroblastoma*, *rhabdomyosarcoma*, *osteosarcoma*, *fibrosarcoma*, and mammary carcinoma cell lines and in human T-cell leukemia virus (HTLV-I)-infected cell lines but not in normal fibroblasts, melanomas, or other carcinomas.[3,6,17,18,21,31,32] In those human cell lines that possess detectable c-*sis* mRNA, a series of polypeptides which are biologically, immunologically, and structurally related to PDGF have been identified in cell lysates and in the conditioned medium (CM).[3,18,26] Moreover, biosynthetic labeling studies have demonstrated that these activities are synthesized and secreted by cells *in vitro*. These mitogens can interact with and can activate the PDGF receptor on their respective producer cells as well as on other PDGF responsive indicator cell lines.[22,24,27,32] Little information is available on the content of PDGF, the expression of c-*sis* mRNA, or the presence of PDGF receptors in fresh tumor biopsies.

Epidermal Growth Factor (EGF) - EGF is an acid and heat stable single chain peptide (Mr 6045) that is acidic (pI 4.5-4.6).[1] In mice the major but not exclusive site of synthesis for EGF is the submaxillary gland. Human EGF (urogastrone) exhibits a 70% amino acid sequence homology to mouse EGF and is biologically but not immunologically indistinguishable from its rodent counterpart in a variety of assays *in vivo* and *in vitro*. In serum and in some tissue extracts, EGF is found in association with a high molecular weight complex (Mr 74,000) composed of two moles of EGF bound to two moles of an EGF-arginine esteropeptidase binding protein (Mr 29,300, pI 5.4). Native EGF is proteolytically processed from a series of high molecular weight precursors (Mr 9000 and 28,000 to 30,000) by the arginine esteropeptidase within cells which are synthesizing and secreting this growth factor.[1,2] EGF is a broad spectrum mitogen since it can stimulate the proliferation of a variety of epithelial and mesenchymal cells through its interaction with specific, high affinity cell surface receptors.[33] The EGF receptor (EGF_R) has been purified to homogeneity from human placenta and from A431 epidermoid carcinoma cells.[33,34] Similar to the PDGF receptor, the EGF_R (Mr 180,000) contains an intrinsic tyrosine-specific protein kinase activity within its cytoplasmic domain. Various domains of the EGF_R have been molecularly cloned and mouse monoclonal antibodies have been generated against some of these regions.[34,35] The gene for EGF (chromosome 4 in humans) in the mouse has also been cloned and the structure of the mRNA for this growth factor has been determined.[36] The size of the mouse EGF mRNA indicates that this species is capable of encoding a large prepro-EGF protein (Mr ~ 133,000) in which the sequence for EGF is found within the carboxy-terminal region. Seven other EGF-related peptides of varying molecular weights may also be processed from the amino-terminal domain of this precursor.[36]

There are several regions within the amino-terminal and the hydrophobic transmembrane domains of this precursor which exhibit partial amino acid sequence homology to portions of the bovine and human low density lipoprotein (LDL) receptor, tissue plasminogen activator, urokinase, and to the c- and v-mos oncogene protein, p37mos.[37,38] Prepro-EGF mRNA, biosynthetically labeled prepro-EGF, and other peptides related to regions within prepro-EGF have been detected in several mouse tissues, and in transformed human fibroblasts.[39,40] The biological function of these cell-derived EGF-like peptides is unknown. Moreover, an EGF-related, glycosylated growth factor (Mr ~ 23,000) has recently been detected in cells which have been infected with vaccinia virus.[41] This growth factor is derived from the viral genome (probably an early virus gene) and exhibits extensive amino acid sequence homology to the transmembrane regions within preproEGF and within αTGF. The vaccinia growth factor can bind to EGF_Rs and may therefore be involved in triggering the proliferation of epidermal cells that is observed following vaccinia infection.

Transformed rodent cells generally exhibit a decrease in their growth requirements for exogenous EGF which in some cases is reflected by a reduction in the number of EGF_Rs on the surface of these cells and parallels an increase in the tumorigenicity of these cells observed in vivo.[6,7,14] However, in some human tumor cells the converse situation may occur.[34] The potential relevance of the EGF_R to transformation comes from the revelation that a region of the EGF_R exhibits a 90% amino acid sequence homology to the avian v-erbB oncogene protein, $p65^{v\text{-}erbB}$.[6,34] This region contains the transmembrane and intracytoplasmic tyrosine-specific protein kinase domains of the receptor, demonstrating that $p65^{v\text{-}erbB}$ actually represents a truncated form of the EGF_R which may possess a constitutively activated tyrosine protein kinase activity.[6,42] In contrast, the c-erbB gene (chromosome 7 in humans) encodes the complete EGF_R protein.[6,42] The tyrosine protein kinase associated with the EGF_R and with the receptors for PDGF, insulin, and insulin-like growth factor-I (IGF-I) are functionally and, in some cases, structurally related to each other and to the src family of tyrosine protein kinases.[42] In addition, a newly discovered oncogene, neu (chromosome 17 in humans), encodes a EGF_R-related 185,000 Mr cell surface glycoprotein (p185neu) and is partially homologous in its nucleotide sequence to the c-erbB gene.[43] Therefore, the neu gene may represent an allele (c-erbB-2) of the EGF_R gene.[44] Amplification, overexpression, and/or rearrangement of the EGF_R gene (c-erbB-1) or the c-erbB-2 allele has been demonstrated in several human cell lines of squamous epidermoid carcinoma cells, breast carcinoma cells, salivary adenocarcinoma cells, pancreatic carcinoma cells, and in biopsies of lung, breast, and brain tumors.[6,7,34,42,44,45] In the case of the EGF_R gene, this amplification is generally reflected in a corresponding increase in the amount of EGF_R mRNA and in an increase in the number of EGF receptors which are expressed on these tumor cells.[34,46-48] The enhanced expression of EGF receptors on these cells may be involved in the progression of some of these tumors in vivo since mouse monoclonal anti-EGF_R antibodies can attenuate the growth of some of these tumor cells in vivo in nude mice as xenografts.[35] Moreover, expression of high levels of EGF_Rs on certain types of lung, brain, bladder, and breast tumors may be useful as a diagnostic and/or prognostic marker.[45,47-50] In the case of breast carcinomas, the level of EGF_Rs is inversely correlated with the estrogen receptor content of these tumors while in breast and bladder carcinomas elevated EGF_R levels can be directly correlated with metastatic propensity.[49,50]

Transforming Growth Factors (TGFs) - TGFs represent a large family of acid and heat stable polypeptides or peptides which have been implicated in the autocrine or paracrine growth of a number of rodent and human tumor cells.[5,7,13,51,52] TGFs reversibly confer upon normal, nontransformed mesenchymal, and epithelial cells several properties associated with the transformed phenotype such as AIG. TGFs consist of at least two functionally and structurally distinct classes of factors, αTGF and βTGF.[7,13,51,52] Alpha TGF (Mr 5,400) is functionally related to EGF since it binds to and interacts with the EGF_R and is a potent mitogen.[53] Mouse, rat, and human αTGFs are immunologically distinct from mouse or human EGF and are only 30 to 40% homologous in their amino acid sequence to either of these EGF species.[53] However, they are 90% homologous to each other. Alpha TGFs may represent an embryonic growth factor utilized in place of EGF.[54] Native βTGF (Mr 25,000) is a dimeric polypeptide composed of two disulfide linked homologous peptide chains (Mr 12,500).[7,52] Beta TGF is structurally distinct from either αTGF or EGF and is not related to PDGF although all of these growth factors can be found in human platelets.[55] Moreover, specific high affinity cell surface receptors which bind βTGF but not αTGF or EGF have been identified and partially characterized on a variety of normal and transformed cells.[52] The genes for rat and human αTGF (chromosome 2 in humans) and for human βTGF have been cloned.[56,57] The size of the mRNAs for α and βTGF indicates that both TGF species are probably derived from larger precursor polypeptides (Mr 24,000 to 43,000) similar to PDGF, mouse EGF, and the human IGFs.[53,56,57] These larger precursor molecules may be embedded within the membrane of cells which are producing these growth factors.[53,56,57] Messenger RNA species for α and βTGF which are capable of hybridizing to specific cDNA probes have been detected in several human tumor cell lines.[56,57] Functionally, α and βTGF cooperate with each other and with PDGF and IGF-I to induce the AIG of some nontransformed indicator cell lines.[55,58]

Both TGF species were originally detected in the CM and cell lysates prepared from avian and rodent fibroblasts transformed by a variety of different retroviruses containing functionally and structurally distinct viral oncogenes (e.g., v-Ki-ras, v-Ha-ras, v-src, v-mos, v-fes, v-fms, and v-abl) and more recently in cells transformed by some DNA tumor viruses (e.g., SV40, polyoma and adenovirus).[7,13,30,51,53,59] Studies using temperature-sensitive or transformation-defective Harvey, Moloney, or Abelson retroviruses have demonstrated that TGFs are cellular gene products whose synthesis is either indirectly or directly controlled by the expression of these viral oncogenes.[7,13,17,31] TGFs have also been identified in the CM and extracts prepared from chemically transformed rat and mouse cells, rodent and human tumor cell lines, and from a spectrum of rodent and human carcinomas.[6,7,13,51,52] The presence of some of these same species, particularly βTGFs, in a variety of nonneoplastic tissues suggests that this class of TGFs may be involved in other normal physiological processes such as wound healing and embryogenesis.[7,54,55] Although TGFs may be necessary along with other growth factors such as PDGF and IGF-II to maintain tumor cell growth, the overproduction and/or enhanced secretion of these activities alone may not be entirely sufficient to initiate neoplastic transformation in vitro.[51,60] Additional biological activities have been ascribed to some of the TGFs. For example, βTGF can function under certain circumstances as a negative growth regulator or chalone for the AIG of some human tumor cell lines and as such may be functionally related to a class of tumor inhibitory factors (TIFs) which can antagonize the biological effects of αTGFs.[61,62] In addition, α and βTGF can induce bone resorption in vitro suggesting that these growth factors which are released by tumor cells may be involved in inducing the hypercalcemia that is observed in vivo in association with

certain malignancies.[63] Alpha and βTGFs have been identified in human urine along with other growth factors such as EGF and as such, quantitative or qualitative changes in TGFs and EGF in body fluids such as urine or serum may be useful as prognostic markers for the presence of occult malignancies.[64,65]

Insulin-like Growth Factors (IGFs) - The IGFs (somatomedins) are a family of low molecular weight (Mr ~ 7,500) peptides which are structurally but not immunologically related to insulin and more distantly homologous to the β subunit of nerve growth factor (NGF).[2,3] In combination with EGF or αTGFs, they are strong mitogenic progression factors in normal rodent and human fibroblasts which have been presensitized with competence factors such as PDGF or FGF.[7,14,66] However, PDGF may not be required as a competence factor in some tumor cells which are expressing elevated levels of the c-, L-, or N-myc genes, since fibroblasts which have been transfected with the c-myc gene that has been linked to a strong viral promoter no longer require PDGF as a competence factor, are able to progress through the cell cycle with EGF alone, and are capable of AIG in the presence of only EGF.[7,14,67,68] The IGFs consist of at least two groups of peptides: a series of basic peptides which include human IGF-I and rat somatomedin C (SM-C) and a group of more neutral peptides consisting of human IGF-II and rat multiplication-stimulating activity (MSA). IGF-I exhibits extensive amino acid sequence homology to rat SM-C while IGF-II is virtually identical to rat MSA.[3] IGF-I predominates in adult sera while IGF-II is found almost exclusively in fetal serum.[3] The major site of synthesis of IGF-I and IGF-II is the liver in adults and in the fetus, although both growth factors are probably synthesized to lesser extents in other tissues and in some cultured fibroblast cells in response to PDGF.[69] The serum levels of IGF-I are regulated primarily by growth hormone while IGF-II levels in the fetus may be controlled by placental lactogen.[3] In serum, IGF-I and IGF-II are associated with discrete high molecular weight binding proteins (Mr 60,000 to 70,000) which exhibit specificity for these two growth factors.[3] Pre-pro and pro-forms of IGF-I and IGF-II (Mr 20,000 to 23,000) have been identified and both growth factors have been cloned from hepatic cDNA libraries.[3,70,71] Using appropriate cDNA probes, it has been possible to elucidate the nucleotide sequence, genomic organization, and chromosomal location of the IGF-I and IGF-II genes. By restriction enzyme analysis of DNA from somatic cell hybrids and by in situ hybridization, the IGF-I gene in humans has been mapped to the long arm of chromosome 12 which also contains the c-Ki-ras-2 proto-oncogene, while the IGF-II gene has been located on the short arm of chromosome 11 in a gene cluster which includes the insulin gene and the c-Ha-ras-1 proto-oncogene.[72] The proximity of the insulin and IGF genes to two ras proto-oncogenes suggests that these gene families may in some way be evolutionarily related and that the expression of the ras and IGF genes in some normal and malignant cells may be coordinately controlled. Interestingly, the gene for the β subunit of NGF is located in a region of chromosome 1 which is adjacent to the other member of the ras family, the N-ras gene.[73]

Human tumor cell lines have been demonstrated to synthesize IGF-I and possibly IGF-II. Biologically active and immunoreactive IGF-I has been detected in the CM obtained from a fibrosarcoma cell line, two osteosarcoma cell lines, renal and transitional carcinoma cell lines, and from several mammary carcinoma cell lines and biopsies.[6,45,74,75] Dot-blot and Northern-blot analysis with an IGF-II cDNA probe has demonstrated elevated levels of IGF-II mRNA in certain embryonal tumors such as a nephroblastomas (Wilms' tumor), a rhabdomyosarcoma, a teratocarcinoma, and a hepatoblastoma.[76] Deletions within chromosome 11 or reactivation of

certain embryonic regulatory genes may be responsible for the overexpression of the IGF-II gene observed within Wilms' tumors and may also relate to the elevated expression of this gene in some normal embryonic tissues.

Specific, high-affinity receptors exist for both IGF-I (Mr α-135,000; β-90,000) and for IGF-II (Mr 250,000).[3,42] The IGF-I and insulin receptors are functionally related to each other since both contain a tyrosine-specific protein kinase which in the case of the insulin receptor is associated with the β subunit (Mr, β-90,000).[6,15,42,45] The structural relationship of the insulin receptor-associated protein kinase to the tyrosine-specific protein kinases of the proto-oncogene proteins of the *src* family of oncogenes has recently been discerned by molecular cloning of the insulin receptor gene.[77] The insulin receptor gene in humans has been localized to a region of the short arm of chromosome 19 in a break-point region which is associated with some pre-B cell leukemias.[78] Furthermore, antibodies raised against $p60^{v\text{-}src}$ can immunoprecipitate affinity-labeled insulin receptors.[79]

Bombesin and Gastrin-Releasing Peptide (GRP) - Bombesin is a tetradecapeptide (Mr 1620) originally isolated from amphibian skin. GRP, a 27-amino acid pancreatic peptide (Mr 2805), is the mammalian counterpart of bombesin.[80] Bombesin or GRP can regulate the level of several gastrointestinal peptide hormones, can stimulate smooth muscle contraction, and can increase gastric acid secretion *in vivo*. Both peptides are immunologically related and bind to the same class of high affinity cell surface receptors on various target cells.[81] Either peptide is a potent mitogen for mouse Swiss 3T3 fibroblasts, human bronchial epithelial cells, and various human small cell lung cancer (SCLC) cell lines *in vitro*.[82]

Human SCLC cells but not large cell lung cancer cells, squamous lung carcinoma cells, or adenocarcinoma lung cancer cells can synthesize and secrete GRP or other bombesin-like peptides.[83] Moreover, peptides which are immunologically related to bombesin have been identified in primary and in some metastatic SCLC tumor biopsies and in certain human gastrointestinal tumors.[84,85] In SCLC cells, GRP or these other bombesin-related peptides are probably involved in regulating the growth of these tumor cells through an autocrine growth mechanism because of the following observations. First, bombesin or GRP can stimulate the AIG of SCLC cells in soft agar. Second, SCLC cells can synthesize and secrete these peptides and third, mouse monoclonal antibodies which were prepared against bombesin and which equally recognize GRP can block the binding of labeled bombesin to its receptor on SCLC cells, can specifically inhibit the clonal AIG of SCLC cells in soft agar, and can attenuate the growth of SCLC xenografts in nude mice.[85]

Conclusions - The recent demonstration that there are highly significant structural, functional, and/or regulatory homologies between certain growth factors or their receptors and specific oncogene or proto-oncogene proteins suggests that these proteins are important in oncogenic transformation *in vitro*.[6,7,16,17] However, several important questions remain unanswered with respect to the potential *in vivo* role of growth factors or their receptors in the etiology of human cancers. First, are qualitative and/or quantitative changes in growth factors or their receptors a secondary consequence of transformation or are these changes actually involved in initiating neoplastic transformation? Second, are there different growth factors or sets of growth factors which regulate the proliferation of normal and neoplastic cells within any given tissue? Third, do normal and malignant cells within any given tissue exhibit the same relative dependency on these activities for growth? Fourth, do the

mechanism(s) of action of these growth factors differ between normal and malignant cells? Fifth, are normal cells capable of synthesizing and/or secreting comparable growth factors as their neoplastic counterparts (and if so, under what circumstances) and can their synthesis or turnover be differentially modulated by other growth factors or hormones? Sixth, is there any unique association with the production of a particular growth factor(s) or the level of expression of its receptor(s) and with a specific type of malignancy or with the degree of differentiation, histopathology, or metastatic aggressiveness within any given lesion? Finally, is there any association between the activation, overexpression, or rearrangement of a particular proto-oncogene(s) in a defined malignancy and the level of production or expression of a specific growth factor or its receptor? The development of specific polyclonal and monoclonal antibodies against growth factors or their receptors and the use of appropriate cDNA probes which are homologous to portions of the genes for these proteins will be efficacious in addressing some of these questions.[45] Moreover, these immunological reagents in conjunction with the isolation or development of natural or synthetic antagonists against these growth factors or enzymes associated with their receptors (e.g., tyrosine-specific protein kinases) should eventually lead to potential diagnostic, prognostic, and/or therapeutic applications.[24,25,35,85-87]

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 17. Chemical Control of Fertility

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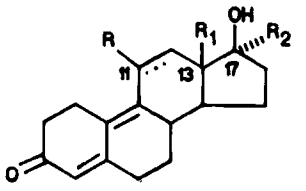
Introduction - Since our 1979 review,[1] advances have been made in the development of agents which interfere with pregnancy by inhibition of progesterone (P) biosynthesis, e.g., epostane, or by antagonism of P at the receptor level, e.g. mifepristone. Significant progress has also been made in the development of prostaglandins (PGs) with improved properties. These subject areas constitute the major portion of this review. The status of research in male contraception is summarized with particular emphasis on the antifertility effects of gossypol. Finally, the antifertility effects of a group of compounds characterized by their structural and mechanistic diversity are described. The use of analogues of luteinizing hormone releasing hormone (LHRH) for both male and female fertility control has been reviewed recently[2] and will not be discussed here.

In addition to comprehensive reviews,[3-5] specialized reviews on menstrual regulation and early post-coital drugs,[6] the immunological approach to fertility control,[7] and long acting contraceptives[8] are available. There has been a decreased emphasis on contraceptive research since 1970.[9] Methods under development or in research which could be available by the year 2000 have been summarized.[10]

Antiprogestins - The potential value of antiprogestational agents as abortifacients and menstrual regulators has been recognized for many years; progress was reviewed in 1979[11] and 1982.[12] Mifepristone (RU 38486, RU 486, 1), binds to P and glucocorticoid receptors,[13] and is related to gestrinone (R 2323, 2), a drug known to possess antiprogestational activity.[14] Termination of pregnancy of 6 to 8 weeks duration by 1, at a dose of 200 mg/day po for 4 days in 9 of 11 women has been reported.[15] In another study, at 100 mg/day for 7 days, 1 terminated early pregnancy in all 10 subjects.[16] In a more definitive study,[17] administration of 1 (25-100 mg bid x 4d) caused only 22 of 36 women to have complete abortions and it was concluded that an increase in efficacy was required in order for the method to compete with vacuum aspiration or PGs.[17] When 1 (25 mg po, bid-qid x 4 d) was administered and a 0.25 mg im dose of the PG analogue, Sulprostone® (26, Table 1) given on the 4th day, 32 of 34 patients underwent complete abortion.[18] The lack of a dose-response relationship in the abortion studies using 1 alone was attributed to insufficient concentration of the drug at the P receptor at the higher doses.[19]

Intravaginal administration of 1 during the mid- to late-luteal phase in the cycling primate, induced early menstruation;[20] a similar effect on menstruation was found after im administration in the estrogen/progestin-maintained ovariectomized monkey,[21] and when given po to humans during human chorionic gonadotropin (hCG)-induced prolongation of the luteal phase, results which were manifestations of the antiprogestational activity of the drug.[22]

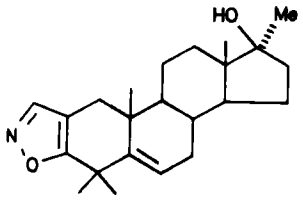


	R	R_1	R_2
1	4-$Me_2NC_6H_4$	Me-	C≡CMe
2	H, $\Delta^{11,12}$	Et-	C≡CH
3	4-$Me_2NC_6H_4$	Me-	-$(CH_2)_3$OH

The antiglucocorticoid activity of 1 was demonstrated in vitro, in rats, monkeys and humans.[13,23-25] 1 interacted weakly with the androgen receptor and did not bind to estrogen or mineralocorticoid receptors.[15] Although it was originally reported to lack progestational activity in nonprimates,[15] 1 may have weak agonist activity in humans,[26] a result which is consistent with the lack of a dose-response relationship in the human abortifacient studies.[18] In estradiol-primed postmenopausal women, 1 induced secretory changes in the endometrium characteristic of a progestin.[26] Similar results were obtained when endometrial biopsies from anovulatory women were examined.[27] Utilizing estrogen independent T47DCO human breast cancer cells in culture, 1 bound to the P receptor and inhibited cell growth as progestins do, but unlike progestin agonists, it did not increase cell surface insulin receptors.[28] It was suggested that 1 has progestin agonist/antagonist properties depending on the response measured.[28]

Although a number of variations of 1 were made at C-11 and C-17, no separation of antiglucocorticoid and antiprogestational activity was achieved except for a modest separation upon inversion of the C-17 ethynyl group from α to β.[29] In contrast, inversion of configuration at C-13 to give a cis C,D ring juncture resulted in a derivative (3) which had 30X less antiglucocorticoid activity in vitro (inhibition of dexamethasone-induced tyrosine aminotransferase) than 1 and whose abortifacient activity in rats was apparently greater than 1.[30]

Steroidogenesis Inhibitors - Problems associated with the development of the closely related 3β-hydroxysteroid dehydrogenase inhibitors 4-7 were species carryover, the ratio of ovarian-placental to adrenal inhibitory activity, and the required suppression of circulating P levels.[31] Azastene (4) was active on day 10 of pregnancy in the rat and on days 50-54 in the monkey but was inactive in women in an hCG model for pregnancy.[31,32] The cyanoketone 5 was completely effective in terminating pregnancy in the rat when administered once on day 10 at 6 mg/kg/po, but was inactive at 1000 mg/monkey on days 50-54.[32] Trilostane (6) and epostane (7) were active in the rat and the monkey and caused a decrease in circulating levels of P in early human pregnancy.[32,33] Epostane exhibited a better ratio of ovarian-placental to adrenal inhibitory activity than 6 when circulating P and cortisol levels were compared.[32] The carbonitrile and epoxide groups were required for optimal interceptive activity in rats and monkeys. Conversion to the β-epoxide caused a marked decrease in activity.[32] Compound 7 terminated pregnancy in 7/7 rhesus monkeys after a single 250 mg sc injection, whereas when given for 5 days po 50 mg terminated pregnancy in 26/34 rhesus monkeys.[34] It inhibited normal and hCG-stimulated P levels during the luteal phase of the menstrual cycle in monkeys[35] and lowered P levels in pregnant women.[36] In an exploratory study, 2 of 3 early human pregnancies were terminated by 100 mg po of 7 administered qid for 5 days.[37]



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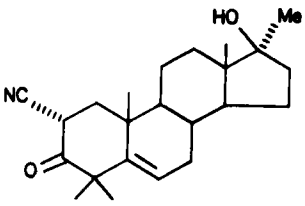

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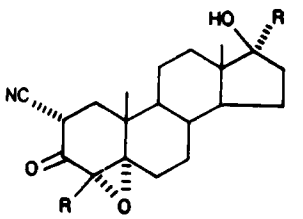
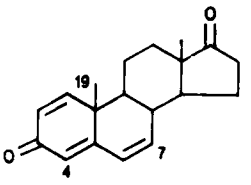


6 R=H, 7 R=Me

Many inhibitors of aromatase are derivatives of andostenedione with modifications at C-4,[38] C-7,[39] or C-19.[40] The proestrus rise in estradiol which induces the LH surge prior to estrus was inhibited by 8[41] and 9[42] resulting in delayed mating. When the administration of 8 was continued in animals which did mate, implantation sites were absent suggesting a postcoital effect.[42]



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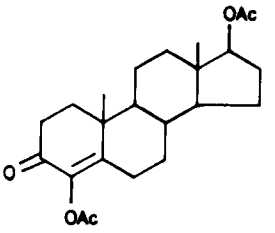

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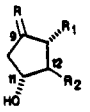
Prostaglandins - Synthetic efforts to find PGs with improved potency and diminished side effects (e.g. gastrointestinal side effects) have continued. Replacement of the Δ^{14} double bond in $PGF_{2\alpha}$ to give 10 resulted in a decrease in antifertility activity in the hamster.[43] Potency was restored by introduction of a phenoxy group in the β side chain as in 11. Maximal antifertility activity was associated with the 15-epi configuration in this series. 16-S-amino $PGF_{2\alpha}$ (12) was 200X more active than $PGF_{2\alpha}$ when given sc in mice on day 17 or 18 and did not cause diarrhea at 10X the effective dose.[44] $PGF_{2\alpha}$ was reported to cause diarrhea at active antifertility doses. Introduction of a fluorine atom at C-12 of $PGF_{2\alpha}$ (13, 12-IIIF) resulted in a 25-fold increase in activity in the hamster antifertility assay believed to be due in part to increased stability toward 15-hydroxy PG dehydrogenase.[45] This change also resulted in a decrease in smooth muscle stimulatory activity. The conformationally-fixed analogues 14 (Z=CH_2,O) of the 17-phenyl-ω-trinor- and 16-phenoxy-ω-tetranor- PGs were usually less active as guinea pig uterine stimulants and more active in causing diarrhea in mice than the open chain compounds.[46] 11-deoxy-PGE_1s were less active than their 11-hydroxy counterparts in the rat uterus stimulation test but potency was restored, for example, by 16,16-dimethylation as in 15 (11-deoxy) and some tissue selectivity achieved.[47] Incorporation of a Δ^3 double bond into 16-phenoxy-17,18,19,20-tetranor-$PGF_{2\alpha}$ (16) caused a 3- to 6-fold increase in antinidatory activity in the rat; 16 was reported to be 1200X as active as $PGF_{2\alpha}$.[48] A corpus luteum site of action for 17 in the rhesus monkey was established since it lowered circulating P levels in the presence of administered hCG.[49] The 17-phenyl group apparently imparts luteolytic activity in this model since 18 was inactive.[50] Compound 19 was also inactive in this test but it had been shown earlier to induce uterine contractions in the monkey. Neither 17 or 19 alone terminated pregnancy on day 28 in the monkey, a time when pregnancy is dependent on both the corpus luteum and the placenta, but together in a single im dose (7.5 mg of 17 and 0.5 mg of 19), pregnancy was terminated in 3/3 monkeys.[50] A luteolytic mechanism may be sufficient for termination of very early pregnancy, but these results suggested the need for an uterotonic component in a PG in order to be effective during the period of the luteoplacental shift. That requirement is borne out by the ineffectiveness of 16-fluoro-13-dehydro $PGF_{2\alpha}$ (20) in terminating pregnancy in the monkey, since the compound had been selected on the basis of a high ratio of luteolytic to smooth muscle stimulatory properties.[51] The time when

a luteolytic drug could be effective in the monkey may be only a few days whereas in the human female it is several weeks.[51] The related 21 (R=F) was 3X as active as 21 (R=H) in the hamster antifertility test and 20-40X less stimulatory of rabbit and rat uterine contractions.[52] Plasma levels in humans of 22 -25 sufficient for induction of first or second trimester abortions after a single vaginal administration were, per ml of plasma, 22, 1-1.5 ng; 23 and 25 (ONO-802, gemeprost, Ewagem®), 200-500 pg; and 24, 15-25 ng.[53] All four PGs were protected from the normally rapid oxidative deactivation at C-15 but underwent oxidative degradation of the carboxyl side chain to nor compounds. The ease of hydrolysis and hamster antifertility activity of three classes of PG lactones were in the order: 1,15>1,11 >1,9.[54] $PGF_{2\alpha}$ 1,15-lactone was effective in terminating early pregnancy in monkeys under circumstances where $PGF_{2\alpha}$ was ineffective.

Table 1 - Prostaglandins

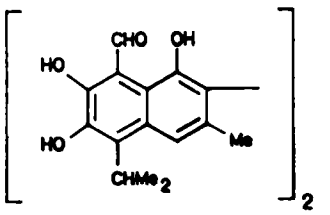


	R	R_1	R_2
10	OH, H	CO_2H	NHCO, OH
11	OH, H	CO_2H	NHCO, O, F, OH
12	OH, H	CO_2H	NH_2, HO
13	OH, H	CO_2H	HO
14	=O	CO_2H	(CH_2), Z, HO
15	=O	CO_2H	HO
16	OH, H	CO_2CH_3	O, HO
17	OH, H	O, CO_2H	HO
18	OH, H	O, CO_2H	HO
19	$=CH_2$	CO_2H	HO
20	OH, H	CO_2H	C≡C, F, HO
21	OH, H	CO_2H	C≡C, R, HO
22	OH, H	CO_2H	HO
23	=O	CO_2H	HO
24	$=CH_2$	CO_2H	HO
25	=O	CO_2CH_3	HO
26	=O	$CONHSO_2CH_3$	O, HO

The most widely used method of pregnancy termination during the first 8 weeks of gestation is vacuum aspiration. The use of PGs for termination of this stage of pregnancy is still in the exploratory stage but results are considered promising.[55] Intravaginal administration of 24 or 25 or im administration of 26 within 3 weeks after the first missed menstrual period were as effective as vacuum aspiration. Side effects included vomiting, diarrhea, and uterine pain but were of lower incidence than observed with the natural PGs. Late first trimester and early second trimester terminations by surgical means were associated with an increased incidence of complications such as cervical injury and hemorrhage. Preoperative cervical dilation by the PG analogues 22, 24, and 25 by non-invasive routes and at sub-abortifacient concentrations resulted in a lower incidence of complications.[55] Medically as opposed to surgically induced termination was the most common method used to terminate mid- to late second trimester pregnancy in four western countries. The technique consists of a two-stage administration of intra-amniotic hypertonic saline and extra- or intra-amniotic PGE_2 or $PGF_{2\alpha}$. Compound 22 is long-acting and thus suitable for single injection but is associated with a high incidence of gastrointestinal side effects; 26 was as effective as 22 and caused fewer gastrointestinal side effects. Intravaginal administration of 24 was as effective as 22 and caused no more gastrointestinal side effects than 26. The best procedure may be pretreatment with one laminaria tent (for cervical dilation) for 12 h followed by im 26.[55]

Male Contraception - Numerous classes of compounds have been identified as potential contraceptive agents for the male yet none has been accepted for human use.[56,57] Spermatogenesis requires normal levels of the gonadotropins, LH and follicle stimulating hormone (FSH). Inhibins are peptides of gonadal origin that selectively inhibit FSH production both in vitro and in vivo.[58-60] Molecular weight estimates of these peptides vary from 3000-30,000 Daltons depending on the source and method of isolation.[58] Inhibin-like peptides isolated from human seminal plasma have been characterized and the smallest, β-inhibin-31, has been synthesized.[59-60]

Several compounds have direct antispermatogenic activity but toxicity and unacceptable side effects have prevented their development as male antifertility agents.[56] Gossypol (27) was most effective in hamsters, followed by rats, monkeys and dogs and was relatively ineffective in rabbits and mice.[61,62] Reversible antifertility effects have been produced in rats at doses of 7.5-30 mg/kg/day po for 6-12 weeks with no significant toxic effects. In man, administration of 27 at a dose of 20 mg/day for 75 days followed by a maintenance dose of 50 mg/week caused sperm counts to fall below 4 million/ml after 3 months in 99% of subjects. Only 75% of men experienced a return to pretreatment levels upon cessation of treatment.[63] Compound 27 acts at multiple testicular sites (late spermatids, pachytene spermatocytes, Sertoli cells) to uncouple spermatozoal oxidative phosphorylation and to inhibit lactic dehydrogenase-X activity.[61] The problems of hypokalemia and irreversible sterility may prevent wide acceptance of gossypol.[63] The racemate of 27 often is isolated from the cotton plant as a 1:1 complex with acetic acid. The antifertility effects of gossypol reside in the (-)-enantiomer.[64,65] In a study of 18 gossypol derivatives substituted with various groups at the hydroxyl and/or aldehyde functions, only one showed modest activity.[62]



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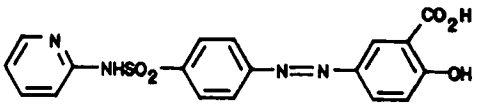
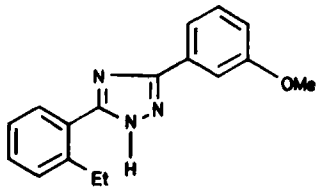
Sulfasalazine (28), a sulfonamide drug used for the treatment of ulcerative colitis, caused reversible infertility in many patients.[66,67] In rats, at 600 mg/kg po for 5 weeks, 28 decreased fertility to 27% of controls. In man, 2-4 g/day for 2



28

months produced changes in sperm density and motility in approximately 60% of subjects without affecting serum gonadotropin or testosterone levels.[68] In rat and man, 28 is metabolized to sulfapyridine and 5-aminosalicylic acid. Sulfapyridine treatment of male rats (321 mg/kg/day x 8 weeks) resulted in significant decreases in the number of fetuses/pregnancy although the pregnancy rate was not affected.

Phenoxybenzamine, an adrenergic blocking agent, inhibited ejaculation in some patients and was evaluated in rats and humans as a potential contraceptive agent.[69,70] Parenteral treatment of male rats with phenoxybenzamine (0.7 mg/kg/5 weeks) produced infertility in 6 of 7 rats which was apparently due to a paralysis of the muscles associated with the sex accessory glands leading to the absence of ejaculation.[69] In studies of men experiencing premature ejaculation, 10-12 mg/day produced azoospermia following orgasm after 2-3 days of treatment and was reversible. Treatment for at least one month did not affect serum gonadotropin and testosterone levels or blood pressure.[70]



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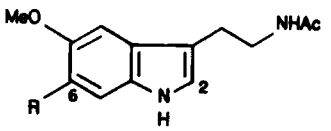
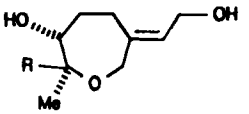


30 R=H
31 R=Cl



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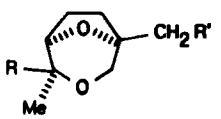


33

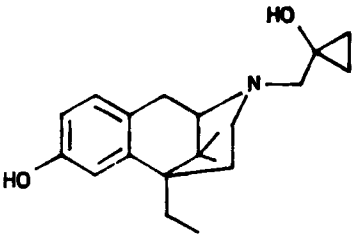
R=$(CH_3)_2C{=}CHCH_2COCH(CH_2)_3$ (with CH_3)

R'=CH_2OH, CHO, CO_2H

Miscellaneous - The 1,2,4-triazole 29 (DL-111-IT) terminated pregnancy at low parenteral ED_{50} doses of 0.4-0.7 mg/kg for 2 to 5 days in the rat, mouse, hamster and dog and was also active in baboons.[71,72] The compound, which has no hormonal or antihormonal activity, is believed to act at the uteroplacental complex,[72] and has been selected for early pregnancy termination studies in women.[73] Melatonin (30, R=H) is metabolized by oxidation at C-2 and C-6. Chlorination of 30 to give 31 changed the plasma half-life in rats from 12-15 minutes to about 27 minutes. Compound 31 was more active than melatonin as an inhibitor of ovulation in the rat presumably because of increased metabolic stability.[74] Melatonin and its 6-chloro derivative inhibited LH release which accounted for the observed inhibition of ovulation.[74] The synthetic conversion of the plant antifertility product zoapatanol 32 to the bicyclics 33 resulted in compounds with greater contragestational activity than 32 in

the guinea pig, a result which suggested that the activity of 32 may be mediated by product(s) of in vivo oxidative cyclization.[75] ORG 13811 (33, R'=CO_2H, R with CHOH instead of CO) caused interruption of pregnancy in mice, rats, guinea pigs, dogs, and baboons in single or multiple doses of 6-60 mg/kg/po depending on the species.[76] Like $PGF_{2\alpha}$, but less active, 33 is both uterotonic and vasoconstrictive, properties which could contribute to the interceptive activity.

Bremazocine (34) and morphine lowered serum LH levels and inhibited spontaneous ovulation in rats.[77] Termination of pregnancy in mice by 35, a mechanism-based irreversible ("suicide") inhibitor of ornithine decarboxylase, resulted from inhibition of the marked activity of ornithine decarboxylase in the embryo.[78] The compound also interrupts pregnancy in rats, rabbits, and hamsters and appears to be well tolerated.[78,79]



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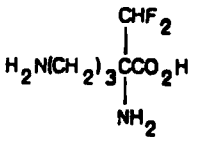

35

Summary - The successful termination of early human pregnancy by the P receptor antagonist mifepristone could be regarded as the most significant development in female fertility control in the last few years. Although preliminary results suggest that this drug may lack sufficient efficacy to compete with existing methods when given alone, the validity of the approach has, nevertheless, been established. In a related and potentially equally important development, epostane, a P biosynthesis inhibitor, caused a fall in plasma P levels in early human pregnancy. Results of definitive pregnancy termination studies are not yet available.

PGs are now used for the termination of mid- to late second trimester pregnancy. Three synthetically modified PGs were clinically advantageous with respect to duration of action, side effects, and stability when compared with PGE_2 and $PGF_{2\alpha}$. Instrumental evacuation for pregnancy termination is increasing, a reflection of a shift from late to early abortion.[80] The percentage of medical inductions among all abortions decreased in the U.S., Canada, and Sweden, again indicative of earlier abortions.[80]

The only accepted means of male contraception are surgical and barrier.[81] Side effects and a significant incidence of irreversibility have impeded the development of gossypol; new derivatives with improved properties have not been reported. There have been no significant developments in the steroid hormone agonist field.

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Chapter 18. Chemical Control of Androgen Action

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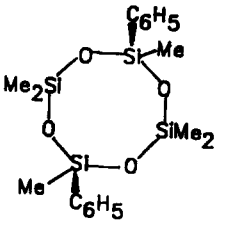
Introduction - Androgens originate primarily from three glands: the testes, the adrenal cortex and the ovary. The primary androgen from the testes is testosterone (T), while the weakly hormonal androgens, dehydroepiandrosterone (DHEA), its sulfate ester and androstenedione, are the primary C_{19} steroids secreted by the adrenal and ovary.[3] Each gland appears to secrete its products with a circadian variation corresponding to some degree with the secretions of stimulatory proteins from the pituitary. The release of these proteins, primarily luteinizing hormone (LH) [also interstitial cell stimulating hormone (ICSH) or lutropin] for the testis-ovary and adrenocorticotrophic hormone (ACTH) for the adrenal, in turn, is controlled primarily by hypothalamic factors, luteinizing hormone releasing hormone (LHRH) and corticotrophic releasing factor (CRF), respectively.[4] Testosterone and its metabolites, estradiol and 5α-dihydrotestosterone (DHT), in a feed-back role are able to influence LH release at the neural and pituitary levels. The release of ACTH is sensitive to the levels of circulating cortisol.[1] Thus, the androgen level in an individual is dependent on a balance of several interrelated stimulatory and inhibitory processes. The end organ response to androgens, including that of feed-back control, is mediated through the androgen receptor (AR) which acts in the cell at the nuclear level to modulate RNA synthesis.[2] The nuclear AR has been found to be associated with only two natural androgens, T or DHT. DHT binds more avidly to the AR than T and, in certain tissues, such as the rat prostate, DHT is the exclusive androgen present in the nuclear isolates. Testosterone is derived from DHEA and androstene-dione via the biosynthetic steps of 3β-ol dehydrogenase/Δ5,4-isomerase and 17β-ol oxidoreductase; DHT is formed irreversibly from T by the membrane bound enzyme 5α-reductase. Genetic deficiencies in androgen-synthesizing enzymes or in the AR result in development of male fetuses with female characteristics.[3] Likewise, female fetuses are virilized by exposure to androgen. Excess androgenic activity is implicated in precocious puberty, male hypersexuality, seborrhea, acne, male pattern baldness and hirsutism. Prostate size and maintenance are androgen dependent, suggesting that interference with this hormone would be a treatment for benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC). Male fertility control would be possible if T-stimulated spermatogenesis could be blocked. Treatments which interfere with androgen action include administration of agents that block regulatory mechanisms, the AR or key biosynthetic steps in the formation of T or DHT. The study of androgen antagonists is over 20 years old and it is only recently that clinical use of these agents has been extensively investigated. This review will discuss recent developments in methods for limiting and abolishing androgen action. Additional information on androgen action[1-5] and androgen antagonists[5-9] is available in several books or reviews.

INTERFERENCE WITH CONTROL REGULATORY MECHANISMS

Estrogens - Estrogens are the natural antagonists of androgens. Increases in circulating estrogen levels generally result in reduced levels and availability of androgens.[10] Estradiol interferes directly with T production at the testicular level.[11] Circulating levels of the testosterone binding globulin increase on estrogen stimulation, causing less free T to be available for cellular uptake.[12] At pharmacological levels, estrogens block the secretion of LHRH and LH and, thus,

the production of T in the testes. This method of chemical castration has been used for years as a non-surgical treatment of PC. The side effects of gynecomastia, loss of libido, liver toxicity and, more seriously, cardiovascular complications are common to estrogen treatment. Diethylstilbestrol (DES), ethinyl estradiol and the estradiol-conjugated mustard, estramustine phosphate (1, Emcyt^{R}), have been used in the treatment of PC.[13] DES only slightly decreases the production of adrenal androgens but may actually potentiate their action in PC by increasing prostate levels of AR.[14] The cyclotetrasiloxane 2 (quadrosilan, Cisobitan^{R}) in a PC trial exerted the same responses, therapeutic and toxic, as other estrogens.[15]



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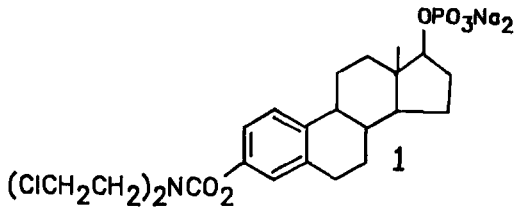

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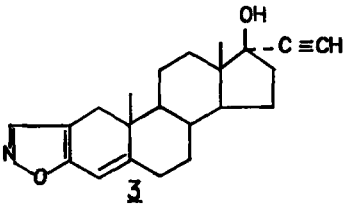

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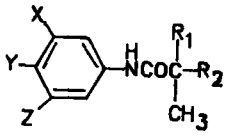
Progestins - Progestational steroids also reduce the effects of androgens. Several modes of action have been postulated the primary mechanism being inhibition of LH secretion.[16-18] In addition, several progestins bind competitively with the AR to reduce the end-organ response[19-20] and some have been shown to modify androgen biosynthesis,[21] metabolism and clearance.[22] The progestins generally appear to be free of the gynecomastia and cardiovascular side effects found with estrogens although loss of libido has been observed. Several progestins have been tested in the clinic for treatment of BPH[23-26] and PC.[27-28] The results, although encouraging, have not been significant enough to replace other means of treatment of these conditions. The gonadotropin inhibitor danazol (3) severely suppressed T levels in a PC trial, but did not affect the course of the disease.[29] The progestational antiandrogen, cyproterone acetate will be discussed below.

LHRH analogs - These agents were reviewed in Volume 20 of this series[30] and the proceedings of an international symposium were published.[31] The fact that continuous, non-pulsatile dosing of the agonist form of these agents provides a mild, reversible means of chemical castration has led to their widespread clinical use for PC. After dosing, the initial surge of T secretion falls to castrate levels. The effect on the course of the disease appears as good as estrogen treatment without the gynecomastia or cardiovascular side effects. Loss of fertility and libido occur as one might expect. Successful trials with buserelin,[32] leuprolide[33] and Zoladex^{R} [34] have been reported. Added benefits appear to be gained by combining LHRH agonists with anti-androgens or androgen biosynthesis inhibitors (see below). Administration of buserelin to male dogs treated daily with percutaneous T or DHT is reported to result in azoospermia without loss of libido or prostatic function.[35] Similar studies in man with nafarelin and the depot androgen, testosterone enanthate, resulted in a lowering of sperm count in 5 of 6 subjects, but azoospermia was not attained.[36]

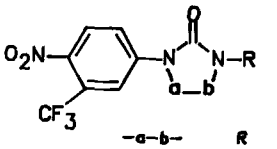
The LHRH antagonist RS-68439, [N-Ac- D-Nal(2)^{1}, D-pCl-Phe^{2}, D-Trp^{3}, D-hArg(Et$_2$)^{6}, D-Ala^{10}]LHRH, rapidly suppressed pituitary and testicular function in rats, dogs and monkeys.[37] Ejaculatory function with continued azoospermia could be maintained by T supplementation in dogs treated with RS-68439.

ANDROGEN RECEPTOR (AR) ANTAGONISTS

Flutamide and Non-steroidal Antagonists - Of the non-steroidal antiandrogens, flutamide (4) has been the most widely studied. It a is "pure" antiandrogen since it appears to have a single mode of action, interference with activated AR-complex formation in target cells. Some of the pharmacology and clinical experience with flutamide has been reviewed.[38-40] Flutamide is metabolized to the more active ∝-hydroxy derivative 5 (Sch 16423).[41] Topical or oral administration of flutamide reduces androgen-dependent sebaceous gland activity in rats and hamsters.[42] The topical effect is not confined to the application area but affects remote sites as well. In humans treated with 4, the sebum secretion rate is affected differently between males (-) and females (+).[43] Although plasma levels of T rise after flutamide dosing as a result of a block to the feedback effect of T, the cellular levels of T and DHT are reduced in androgen target tissues.[44] Clinical studies for treatment of BPH with flutamide were inconclusive and pointed out the need for better indices of improvement in this disease.[45] Extensive trials for treatment of PC with flutamide have been conducted.[39,40,46] In general, results equivalent to those seen with castration or DES treatment were found. Side effects were primarily breast tenderness and gynecomastia. More positive results for PC treatment have been attained with flutamide used in combination with LHRH agonists or orchiectomy.[47] The anti-androgen reduces the effect of the early T surge caused by the agonists and also neutralizes the effect of circulating non-testicular androgens. The objective response rate was very high relative to castration alone. Side effects were hot flashes and diminished libido.



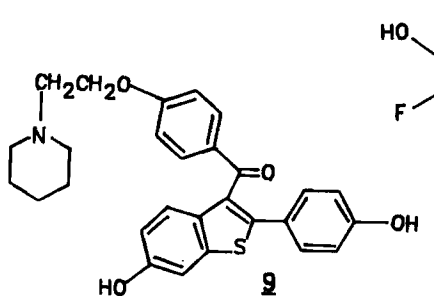
	X	Y	Z	R_1	R_2
4	H	NO_2	CF_3	H	CH_3
5	H	NO_2	CF_3	OH	CH_3
6	Cl	Cl	Cl	OH	CH_2Cl



	-a-b-	R
7	$-COC(CH_3)_2-$	H
8	$-(CH_2)_2O-$	CH_3

The related anti-androgen 7 (RU 23908, Anandron^R) which has pharmacological properties similar to flutamide,[48] effectively blocks the trophic effects of adrenal steroids in castrate- or DES-treated rats.[49] In a human PC study 7 with a LHRH agonist or with orchiectomy, resulted in significant improvement symptoms over prior therapy. However, in prolonged studies, 7 caused visual problems and was replaced with flutamide.[47] Topical treatment of hamsters with 8 (RU 22930) was as effective as 4 or 7 in blocking T-stimulated flank organ growth but when given subcutaneously, 8 showed little effect on T-stimulated prostate growth.[50] The tetrachloro-∝-hydroxyisobutyranilide 6 (AA560) resembles flutamide in its action.[51] A series of potent anti-androgenic hydroxyflutamide analogs have been prepared and compared with steroidal and non-steroidal anti-androgens for in vitro and in vivo activity.[52] No correlation of biopotency and receptor binding affinity (RBA) could be determined. However, some analogs at a high dose stimulated castrate rat prostatic growth indicating that agonist activity can be obtained with non-steroidal agents. Anti-androgenic activity can be retained when an olefin, acetylene or cyclopropyl group is substituted for the amide moiety of such anilides.[53] Non-steroidal anti-androgens outside the flutamide class are rare. The antiulcer agent cimetidine, is a weak AR antagonist which occasionally induces gynecomastia in man.[54] When given to hirsute women over a 3 month

period, cimetidine showed a beneficial effect on hair growth.[55] The anti-estrogen raloxifene (9, LY-156758) also has androgen antagonist properties and has potential for use as a dual-acting agent in BPH.[56] The DES-like anti-androgen bifluranol, (10) showed some desirable effects for treatment of BPH.[57] The phthalimide 11 (DIMP) is a weak anti-androgen which was active in standard animal assays.[58]



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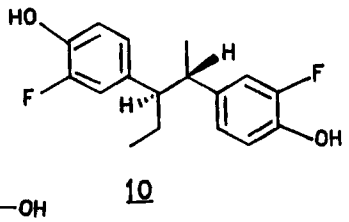

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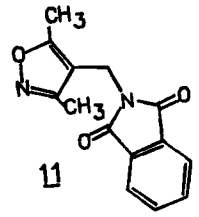
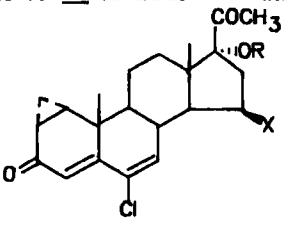


11

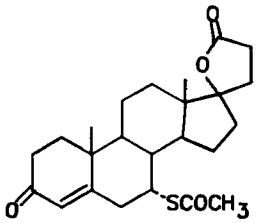
Cyproterone Acetate (12) - The most thoroughly studied androgen antagonist is cyproterone acetate. Systematic studies over the last 24 years have resulted in its application for treatment of most androgen related disorders. Several reviews are available.[59-61] Whereas the parent alcohol, cyproterone (13), limits androgen action primarily by interference at the level of the AR; the acetate 12, a potent progestin, blocks the AR, acts as a gonadotropin inhibitor and enhances the clearance of testosterone.[62] In man, 12 is metabolized to its 15β-hydroxy analog 14, which can occur at levels twice that of 12 in plasma[63] and which retains the androgen antagonist activity of 12 but is a less potent progestin.[64] Because of its potential for fetal feminization 12 is often administered in combination with ethinyl estradiol as a contraceptive regimen. This combination has found extensive use as a treatment for hirsutism in women. Effects on hair growth (hirsutism or alopecia) in females require 3-9 months to develop[65] while noticeable effects on acne[66] and seborrhea appear earlier and seem more sensitive to treatment. Treatment of acne in males with oral 12 is effective but side effects of gynecomastia, loss of libido and spermatogenic defects have been observed.[67] When topically applied, 12 is less effective and is associated with systemic effects.[68] Trials of 12 in BPH[69-70] and PC[28] have shown qualitative degrees of success but have not gained wide acceptance. More recent studies in which 12 is combined with DES[71] or buserelin[72] have given preliminary indications of being better treatments for PC. Some success has been observed in male breast cancer treated with 12.[73] Precocious puberty in boys and girls has been treated with 12 although interference with adrenal function was seen in this age group.[59] Suppression of hypersexuality in men with 12 is more effective than castration.[74] Spermatogenesis is inhibited by 12, but its use for male contraception does not seem promising.[75]



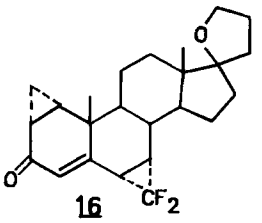
	R	X
12	$COCH_3$	H
13	H	H
14	$COCH_3$	OH

17β-Hydroxysteroids - A number of 17-oxygenated steroids have shown androgen antagonist activity. Probably the most thoroughly studied is the aldosterone antagonist spironolactone (15). In early studies, development of gynecomastia occurred in patients after prolonged use. A dual mode of action, inhibition at the AR[76] and interference with the 17-20 side-chain cleaving enzyme,[77] is thought to provide the primary means of androgenic deprivation. When 15 was given to castrated PC patients, plasma levels of adrenal androgens were reduced.[78] In several studies, a beneficial effect for 15 in treating hirsutism and acne in women[79-81] was found, although in a comparative study, 15 was not superior to 12.[82] Additional benefit may be derived by using a combined regimen of 15 with

an oral contraceptive or dexamethasone.[83-84] Topical application of 15 has shown a local anti-androgenic effect on the male hamster flank organ.[85] The spiroether 16 (MK-316), which combines some structural features of 12 and 15, is effective in reducing the size of dog prostate.[86]



15 9



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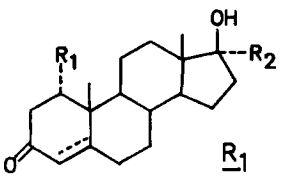
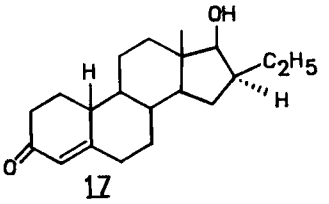


		R_1	R_2
18	Δ4	H	n-C_3H_7
19	5α-H	CH_3	n-C_3H_7
20	5α-H	CH_3	H

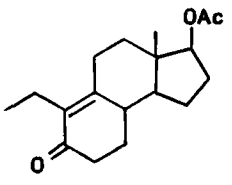
By manipulating the bulk at the 16-position of 19-nortestosterone, optimal androgen antagonism and minimal trophic effects were obtained with the 16β-ethyl derivative 17 (TSAA-291, oxendolone). Reviews and a comprehensive report of studies with 17 have appeared.[87-89] This material binds to the androgen receptor with an apparent K_i of 7 x 10^{-8}M and is a modest inhibitor of the enzyme 5α-reductase.[90] The effects of 17 on gonadotropins is a typical, being slightly inhibitory in male and stimulatory in female rats. In the dog, no effect on T levels is seen but the prostate is reduced in size. In a number of clinical studies for treatment of BPH with 17, injected weekly, improved symptoms with a minimum of side effects have been observed.[91-93]



17

Additional bulk at the 17α-position of testosterone as, for example, with the 17α-propyl derivative 18 (topterone) resulted in an effective androgen antagonist.[94] This material blocks the actions of T and DHT by topical and systemic administration. In recent experiments, the 17α-propyl analog 19 (SH-434) of the potent androgen mesterolone 20 was only locally active as an anti-androgen on topical administration to hamsters.[95] When applied topically to humans with acne and seborrhea for an average of 3 months, 19 effectively reduced sebum excretion by 23-60% and improved symptoms in 11 of 13 patients.[96] No effect on serum hormone levels was found; 19 is in Phase 1 clinical trials.[143]

A number of 17β-hydroxy-des-A-steroids have been assayed for androgen antagonist activity.[97] As was the case with other pure anti-androgens, their interaction with the AR was more labile than that of T or DHT. For AR binding, the equivalent of the C-1 of a steroid must be present in these molecules and planarity of the C-10 to C-12 edge increases activity. An anti-acne clinical candidate developed from this series, 21 (RU 38882), given orally or s.c. to rats, had less than 1/25 the androgen antagonist activity of 12, but on topical application it was 100-fold more active in reducing sebaceous elements in the same animal without showing systemic effects.[98] A des-D-19-nortestosterone analog 22 (Ro 5-2537) topically reduced sebum production in humans better than some steroidal anti-androgens.[99]



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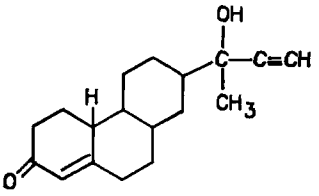

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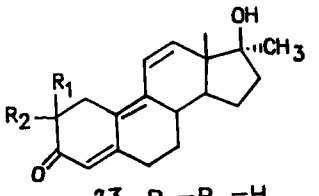
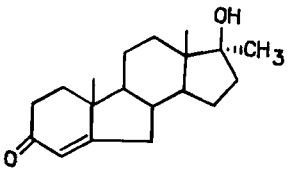


23 $R_1=R_2=H$

24 $R_1=R_2=CH_3$

Addition of methyl groups alpha to the carbonyl of the potent androgen 23 (R 1881) converted the compound into the anti-androgen 24 (R 2956). This material (24) which blocked androgen action in rats and dogs,[100] was not a 5α-reductase inhibitor but exhibited weak gonadotropin inhibition in female rats. B-nor-Methyltestosterone (25) is an androgen antagonist which has shown a beneficial clinical result for acne and hirsutism after oral dosing.[101] Weak anti-androgenic activity in mice has been recently reported for the cyclopropyl analog 26 of *testosterone*.[102]



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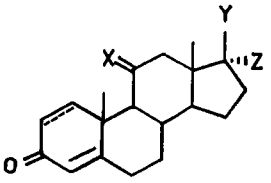
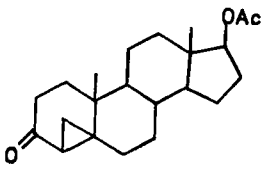


	X	Y	Z
27	α-OH	$COCH_3$	H
28	Δ1, O	$SOCH_2Ph$	Cl
29	α-OH	CO_2H	OH
30	Δ1, β-OH	CO_2H	OH

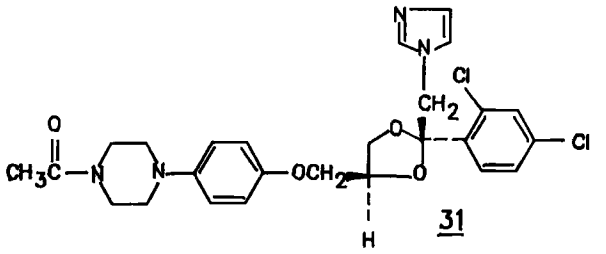


26

11-Oxygenated Steroids - Several steroids which topically reverse androgen stimulated skin conditions but have shown little or no anti-androgenic effects on systemic administration include the 11-oxygenated steroids 27-30. Long-term studies in humans indicated that sebum production and hair loss could be reduced by treatment with 27.[103] The 11-oxosteroid 28 was the best of several such 17-sulfinyl steroids in blocking androgen-regulated hamster flank organ growth and lipid synthesis.[104] The two carboxysteroids 29 (Org 7294) and 30 (Org 7476) are not 5α-reductase inhibitors, but reduce T- and not DHT-stimulated sebaceous gland growth.[105]

BIOSYNTHESIS INHIBITION

17,20-Desmolase - Ketoconazole (31), an antifungal drug which prevents ergosterol biosynthesis in fungi,[106] is a potent inhibitor of the cleavage reaction which converts 17α-hydroxy progesterone to androstenedione, the penultimate step in T biosynthesis.[107] Treatment of humans with 31 resulted in an immediate decrease in C_{19} steroids but feedback effects tended to reverse this process.[108] At constant,



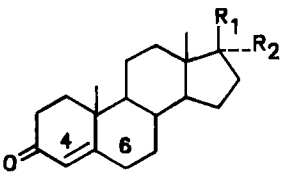
31

high doses, a reduced androgen level was maintained and led to beneficial effects in PC.[109] The advantage with this treatment is that both adrenal and testicular androgen synthesis are blocked with only moderate effects on corticoid production. The use of 31 with the LHRH analog Zoladex^{R} in PC patients who failed to respond or relapsed on conventional hormone therapy has shown some promise.[110] The successful use of 31 in precocious puberty has been reported.[111]

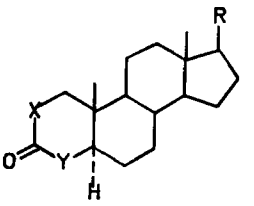
3 β-ol Dehydrogenase - Although inhibition of this enzymic step could prevent the conversion of DHEA into androstenedione and, thus, into T it is of more consequence in the production of progesterone from pregnenolone (see Chapter 17 of this volume).

5 α-Reductase - In many androgen target tissues, most notably the prostate and skin, T is largely converted to DHT, which is the trophic hormone in these tissues. Agents which prevent DHT formation should be selective anti-androgens which would not interfere with independent actions of T on muscle or in the brain. In early in vitro studies with human skin 5 α -reductase, 3-keto-Δ^4 steroids with an oxygen-containing 17 β-side chain were effective enzyme inhibitors.[112] Additional studies with the non-hormonal 17 β -carboxy-4-androsten-3-one (32) on the female hamster flank organ or in the castrate mouse indicated that T- but not DHT-stimulated growth could be attenuated.[113] Progesterone (33) is a potent 5 α -reductase inhibitor which is extensively metabolized in the skin. When topically applied, 33 selectively and locally blocks T and not DHT stimulation in hamsters.[114] On human skin, 5 α -reductase is completely blocked by topical 33, but skin areas remote from the application site may be affected.[115] A reduction in sebum excretion was seen in women but not men in one study with topically applied 33,[116] while in another study 85% of the men responded with decreased excretion.[117]

A structural activity (SAR) study of 4-azasteroids as inhibitors of rat prostatic 5 α-reductase has been reported.[118] Optimal in vitro activity resides with 4-methyl-3-oxo-4-aza-5 α -androstanes substituted in the 17 β -position with a semipolar group. Examples of these compounds have been found to inhibit the 5 α reductases of rat prostate, pituitary, hypothalamus, liver and interstitial cells, human prostate and skin, dog prostate and hamster sebaceous gland.[119-125] Oral or s.c. administration of 4-MA (34) to rats decreased the DHT content of the prostate while T levels rose.[126] In castrate rats 34 was more effective in blocking T stimulated prostate growth than that stimulated by DHT. Reduction in dog prostatic size has been demonstrated with 34.[127-128] The rate of growth of a rat PC tumor was slowed to that equivalent to castration by administration of 34 or 35.[129] Azasteroids 34, 35 and 36 caused fetal feminization of offspring of treated pregnant rats.[130-132] Azasteroids unsubstituted on the ring nitrogen have very low affinity for the AR.[133] The oxazasteroid 37 is a poorer 5 α-reductase inhibitor than the parent azasteroid.[134] The 4-chlorosteroid 38 was the best (K_i=95nM) of several 2- and 4-substituted steroids for inhibition of rat prostatic 5 α-reductase.[135]

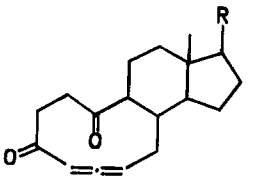


	R_1	R_2	Other
32	CO_2H	H	
33	$COCH_3$	H	
38	$CH(CH_3)CH_2OH$	H	4-Cl
39	$COCH_3$	H	$6=CH_2$
40	$COCH_3$	OAc	$6=CH_2$



	X	Y	R
34	CH_2	NCH_3	$CONEt_2$
35	CH_2	NCH_3	$CH(CH_3)CO_2Na$
36	CH_2	NH	$CON(i\text{-}C_3H_7)_2$
37	O	NCH_3	$CH(CH_3)CH_2OH$
43	CH_2	CN_2	$CH(CH_3)CH_2OH$

A number of irreversible inhibitors of 5α-reductase have been reported. The addition of a 6-methylene group to 3-keto-Δ^4 steroids maintained or increased the in vitro 5α-reductase activity of the parent compound.[136-137] The pregnanes 39 and 40 have shown a time-dependent inhibition of the enzyme.[137] When given parenterally 40, reduced rat prostate growth.[138] The 5,10-secosteroids 41 and 42, also apparent Michael acceptors, appear to be irreversible inhibitors of rat epididymal 5α-reductase.[139] When given topically, 42 blocked the effects of T on the female hamster flank organ.[140] The 4-diazo-5α-steroid MDL-18341 (43) is a selective irreversible inhibitor of 5α-reductase (K_i = 35 nM) with no affinity for the DHT-metabolizing enzyme 3α-hydroxysteroid oxidoreductase.[141] In castrate rats, 43 blocked T but not DHT effects on sex accessory glands.[142] The T effect on growth of the levator ani was not antagonized by 43.



41 R=O
42 R=$COCH_3$

Conclusion The development of agents which will limit or block androgen action is reaching new levels of refinement. The use of LHRH analogs, alone or in the presence of androgen antagonists or synthesis inhibitors, now provides a highly effective and safe alterntive to surgical castration in the treatment of PC. Idiopathic hirsutism often responds favorably to anti-androgen treatment but development of agents for the treatment of non-life threatening ailments such as skin disorders or BPH has been slow because of the potential for hormonal side effects, slow or erratic onset of action, alternative medical or surgical methods and a lack of appropriate experimental models. The use of 19 and 21, which show local topical effects with negligible systemic activity in animal models, is an example of recent attempts to develop treatments which are safe and selective in their action. Similarly, the new 5α-reductase inhibitors afford a class of compounds which may be free of some side effects associated with classical anti-androgens. Such targeting of inhibitors to specific tissues or enzymes has the potential to provide therapeutic agents which can be used safely on a much larger scale than was possible in the past.

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Chapter 19. Approaches to Drug Intervention in Atherosclerotic Disease

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Introduction - The progression of atherosclerotic disease from fatty streak to fibrous plaque occurs over decades in most individuals and is initiated or aggravated by risk factors including hypertension, personality traits, genetics, smoking, obesity, age and sex. However, only hypercholesterolemia has been shown in large scale, double-blind, placebo-controlled clinical trials to be a definitive and sufficient cause for coronary heart disease (CHD). Not only is there a causal relationship between high serum levels of cholesterol (C) and CHD, but reduction of plasma C, in particular low density lipoprotein-cholesterol (LDL-C), can in certain patients result in a decrease in the degree of stenosis of coronary arteries. Since the economic impact of CHD in the United States alone is estimated at $78.6 billion for 1986 (or approximately double the figure for 1978), and that almost 1 million people will lose their lives to heart attack, stroke and other vascular diseases this year, there is an even greater need for new and better approaches toward pharmacological intervention. This review will discuss recent advances in the regulation of plasma lipids, therapeutic approaches based on cellular C metabolism in specific tissues, and lastly, possible points of metabolic regulation not primarily involving lipid and lipoproteins.

Abnormalities of Lipoprotein Metabolism - The most common monogenic hyperlipidemia, familial hypercholesterolemia (FH), an autosomal dominant trait that affects both homozygotes and heterozygotes, was discussed in the previous review by Prugh, et al.[1] FH subjects can present with either the Type IIa or IIb phenotype (Table 1). Because of their lack of functional receptors, it is generally believed that FH homozygotes do not respond to dietary or drug treatment whereas heterozygotes with about half the number of receptors do respond.

The majority of non-FH hypercholesterolemic individuals probably have a reduced number of hepatic LDL receptors due to such environmental factors as a high dietary intake of saturated fat and C.[2] Thus, the LDL receptor has been implicated in virtually all types of hypercholesterolemia (mild, moderate and severe)[2,3] even though its discovery was based on the extremely rare FH homozygous condition.

Another possible cause of elevated LDL concentrations is familial combined hyperlipidemia (FCHL), probably the most frequent lipid disorder.[4] FCHL subjects may or may not have elevated triglycerides (TG) and thus can present with variable phenotypes (IIa, IIb, IV). Several phenotypes are present in the family members. This is in contrast to familial hypertriglyceridemia in which hypertriglyceridemia, but not hypercholesterolemia is present in family members (i.e., only Type IV). It is likely that FCHL is due to an overproduction of apolipoprotein B (apo B) with or without overproduction of VLDL-TG.[5,6]

Table 1 Fredrickson Lipoprotein Phenotypes

Type	Elevated Lipoprotein	Plasma Cholesterol	Plasma Triglyceride
I	Chylomicrons	Normal or Increased	Markedly Increased
IIa	Low density lipoproteins (LDL)	Increased	Normal
IIb	Very low density lipoproteins (VLDL)	Increased	Increased
III	Abnormal β-VLDL	Increased	Increased
IV	VLDL	Normal or Increased	Increased
V	Chylomicrons VLDL	Increased	Increased

Rare apolipoprotein disorders have been summarized.[5-15] Type III hyperlipoproteinemia is characterized by hypertriglyceridemia, hypercholesterolemia, CHD and/or peripheral vascular disease, and is due in part to the presence of an abnormal VLDL (β-VLDL) caused by the inheritance of a gene coding for an abnormal form of apolipoprotein E (apo E-2). This apo E-2 does not bind normally to the LDL receptor or the chylomicron remnant receptor, preventing normal processing of both intestinal and hepatic lipoproteins, and resulting in the formation and accumulation of remnant lipoproteins or β -VLDL. However, only 1 in 50 individuals with apo E-2 develop the clinical symptoms noted above, so other factor(s) must also play a role in the etiology of Type III.[4] For example, it is known that diets rich in fat and cholesterol cause the appearance of β -VLDL-type particles,[7,8] and such diets may be even more atherogenic in apo E-2 subjects compared to normal individuals. Apo E, lipid transport and Type III hyperlipoproteinemia have recently been reviewed.[7-13]

Various systemic diseases such as diabetes and hypothyroidism can produce conditions that resemble the primary hyperlipoproteinemias.[15] Other "secondary hyperlipidemias" are found in patients with liver or kidney disease, in obese individuals, or may be due to administration of drugs such as antihypertensive agents. Once the secondary hyperlipidemias have been ruled out by appropriate laboratory tests, the diagnosis of primary hyperlipoproteinemia can be made and dietary or pharmacologic intervention can be recommended.

A new phenotype, hyperapobetalipoproteinemia, in which LDL concentrations are normal but the amount of apo B in LDL (LDL-apo B) is elevated,[5] is thought to be associated with CHD and may be present with or without elevated TG. Recently, several forms of altered lipid transport termed "lipoprotein overproduction disorders," the underlying

defect of which is excessive synthesis of apo B, have been described.[16] By this criterion, hyperapobetalipoproteinemia occurs when overproduction of VLDL-apo B leads to increased conversion of VLDL to LDL (increased apo B/C ratio in LDL).[6,16-17] Clearly, understanding more about the regulation of apo B synthesis could offer a therapeutic approach in the future for a variety of lipoprotein disorders.[18,19]

Pharmacologic Intervention - Lipid Regulators - In view of the generally accepted concept that LDL and HDL represent positive and negative risk factors, respectively, in atherogenesis, the term "lipid regulator" has to some extent replaced "hypolipidemic agent" for those drugs designed to treat lipid disorders. Emphasis on C distribution among lipoproteins is also evident with the use of various C ratios as atherogenic indices (e.g. HDL/total C, HDL/LDL, HDL/VLDL+LDL).[20,21] Drugs which only elevate HDL cholesterol are also under development.[22] Based on the Framingham Study,[23] the ideal therapy was thought to be one which would raise HDL as it lowered LDL. Recent published reports[24-32] have suggested that the only question remaining is not whether but how to treat.[33] Niacin, the oldest lipid regulator, is still recommended as the drug of choice for virtually all primary lipid disorders.[34,35] The discussion below concentrates on newer agents and includes only the most recent data for the established lipid regulators such as niacin.

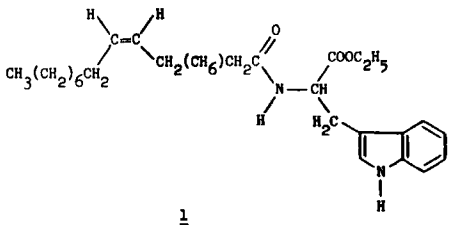
Bile Acid Sequestrants - Cholestyramine and colestipol are anion exchange resins which bind bile acids in the intestinal lumen. The therapeutic effects of this interruption of the enterohepatic circulation include upregulation of hepatic LDL receptors, lowering of plasma LDL and slight increases in HDL levels in Type II patients.[36-38] Although disadvantages of resin therapy are well documented,[4,36,38-43] in recent prospective, placebo-controlled, double-blind trials, cholestyramine significantly lowered the incidence and progression of CHD in hypercholesterolemic males.[24-27] As a result of these studies, efforts to lower LDL and raise HDL appear to be worthwhile in terms of CHD risk. More dramatic lowering of LDL (50% reductions instead of 20% as for cholestyramine) has been advocated[32] and may be possible with new resins possessing greater affinities for bile acids or with various combination treatments.

Recently cholestyramine treatment has been reported to result in the accumulation of LDL with altered composition. Large, cholesteryl ester-rich LDL particles are preferentially removed and smaller, more dense, cholesteryl ester-poor particles remain.[44] Cholestyramine in combination with an agent which normalizes LDL composition, such as gemfibrozil, has been suggested.[45]

Inhibitors of Cholesterol Absorption - Agents which act in the intestinal lumen to block or slow the entry of C into the mucosal cells include neomycin,[46] various surfactants (AOMA or surformer,[47] BEP,[48] Poloxalene 2930[49]), plant sterols,[50] steroids (diosgenin,[40,51] saponins[52]), sucrose polyester[53] and synthetic glycosides.[54] Of these, neomycin lowered LDL as effectively as cholestyramine and may actually be preferred due to its lower cost and ease of administration.[55] Like the resins, its primary mechanism of action is upregulation of hepatic LDL receptors resulting in enhanced clearance of both LDL and VLDL remnants and decreased LDL production.[56]

Fatty acid amides are another group of compounds which inhibit C absorption.[57] Compound 57-118 (1) lowered C absorption in rabbits by

65%,[58] and compound 58-035 (2) decreased C transport in lymph fistula rats.[59] These acyl amides, which are thought to inhibit C absorption by inhibition of intestinal C esterification by acyl-CoA:cholesterol acyltransferase (ACAT), are probably not systemically absorbed,[60] do not inhibit TG absorption[59] or retinol esterification[61] and, like neomycin[56] and sucrose polyester,[62] also inhibit the absorption of endogenous (biliary) C.[59] Thus far, no clinical data are available for 1 or 2; clinical data for the earlier linoleamides moctamide[63] and melinamide[64] show decreases in serum C generally $\leq$ 10% in dyslipidemic subjects. Octimibate (3)[65] and CL-277,082 (4)[66] are ACAT inhibitors which appear to have direct anti-atherosclerotic effects on arteries of C-fed animals.



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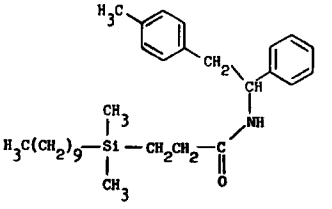

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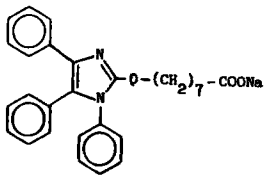

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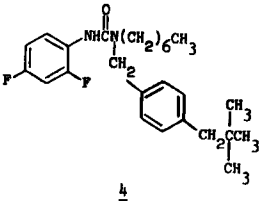

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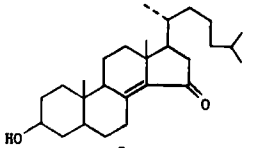
HMG-CoA Reductase Inhibitors - The mevalonate analogs compactin and mevinolin previously reviewed continue to be studied as agents for the treatment of hypercholesterolemia;[38,39,41,43] their isolation and actions were also, recently reviewed.[39,67] Recent findings show that mevinolin does not deplete vital stores of body C,[68] does not affect adrenal function[69] and is efficacious in even moderately hypercholesterolemic individuals.[70] In a large, multicenter trial of mevinolin in FH heterozygotes, decreases in LDL-C of 42% were achieved.[71] Compound 5 is another inhibitor of HMG-CoA reductase being developed which may possess advantages over other oxysterols since it is a precursor of C.[39] It was recently shown to lower LDL (-46%) and elevate HDL (+57%) in rhesus monkeys.[72] The HMG analogs 6[73] and 7[74] have also been described.



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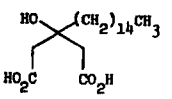

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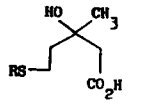
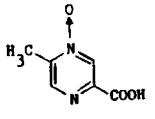


R = CoA, pantetheine

7

Nicotinic Acid (Niacin) This lipid regulator has been used since 1955 for virtually all types of dyslipidemias[34,35] despite noncompliance due to side effects.[55] In the Coronary Drug Project[75] nicotinic acid reduced the incidence of nonfatal myocardial infarctions (MI), but the incidence of atrial fibrillation and other arrhythmias was increased.[39] Unlike HMG-CoA reductase inhibitors, C absorption inhibitors or bile acid sequestrants, nicotinic acid has no effect on the clearance of LDL by the liver. Its major action is to decrease hepatic VLDL synthesis by

inhibition of adipose tissue lipolysis and thus to limit fatty acid mobilization.[76,77] Analogs of nicotinic acid are also in clinical use.[39,77,78] Acipimox (8) elevated HDL in both Type IV and Type II subjects but did not lower total or LDL-C in Type II patients (IIa and IIb).[79] Other recently reported analogs still in preclinical stages are glunicate (LG 13979,9)[80] and KCD-232 (10).[81]



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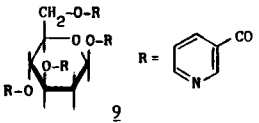

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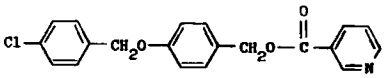

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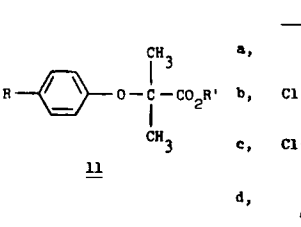
Probucol - Probucol modestly lowers total C and LDL-C by increasing LDL catabolism,[82] primarily by nonreceptor mechanisms.[83] It has no effect on TG and consistently lowers HDL-C by decreasing the synthesis of HDL apoproteins A I and A II.[84] Probucol is lipophilic and alters the metabolism of lipoproteins.[83] In monkeys fed high-fat diets, high blood levels of probucol were associated with cardiotoxicity.[85] In some studies, no change in serum C or LDL-C levels with up to eight weeks of treatment was found,[86,87] but other reports showed that probucol caused regression of xanthomas and lowered LDL-C levels in FH homozygotes.[88-90]

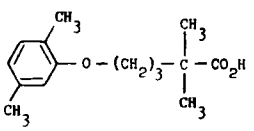
Chlorophenoxyacetic Acid Derivatives - Clofibrate (11a) and the other structurally related derivatives bezafibrate (11b), fenofibrate (11c) and ciprofibrate (11d) may act by stimulation of LPL;[91] TG lowering is the major pharmacologic effect. All of these drugs have been reported to elevate HDL. However, in direct comparative studies both 11d[92] and 11b[93] elevated HDL to a greater extent than 11c in Type II patients. In hypertriglyceridemic subjects, 11c elevated only HDL_3 [94] while 11b elevated only HDL_2 without accompanying changes in apo A I or A II metabolism.[95] Bezafibrate is the only derivative shown to increase receptor-mediated clearance of LDL in hypercholesterolemic individuals.[96] Fenofibrate increases the fractional catabolic rate of LDL-apo B in heterozygous FH subjects which is also consistent with enhanced receptor activity.[97] Eisenberg[98] has postulated that hypertriglyceridemia alters the composition and hence metabolism of all lipoproteins, thus contributing to atherogenesis. For example, LDL from hypertriglyceridemic subjects is less effective than normal LDL in interacting with the LDL receptor, and this defect is reversed by bezafibrate therapy.[99]



	R	R'
a,	Cl	CH_3CH_2
b,	Cl-⟨⟩-$CONH(CH_2)_2$	H
c,	Cl-⟨⟩-CO	$(CH_3)_2CH$
d,	$Cl_2C(CH_2)C-$	H

11

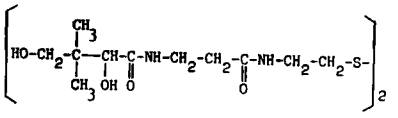
Gemfibrozil - Gemfibrozil (12) is often considered separately from clofibrate and its congeners in view of its structural differences.[1] In contrast to 11a and 11b , 12 elevated HDL-C and lowered liver C concentrations in C-fed rats.[100] Gemfibrozil also prevented decreases of apo E in C-fed rats,[101] a property not shared by 11a-d.[102] In hypertriglyceridemic subjects, 12[103] but not 11b[95] increased the synthesis (and plasma concentration) of apo AI and AII without



12

changing the catabolism of these HDL peptides. Unlike 11c,[94] 12 decreased LDL-apoB production in hypertriglyceridemic patients,[104] although both drugs tended to normalize LDL composition.[94,104] 12 also decreased the rate of VLDL-apo B and intermediate density lipoprotein (IDL)-apo B synthesis in Type III subjects.[105] In a direct comparison in hypertriglyceridemic individuals, 12 both decreased the production and increased the clearance of VLDL-TG whereas 11a only affected clearance.[106] The ongoing 5-year Helsinki Heart Study is designed to test whether all of these lipid-regulating effects will prevent CHD in (primarily) Type II male subjects.

Pantethine - Pantethine (13), a pantothenic acid derivative presently marketed in Japan for dyslipidemia, lowered C and TG concentrations and elevated HDL in Type IIb subjects.[108] Stimulation of LPL in rats[109] and elevation of HDL in rabbits[110] have been reported, consistent with the clinical findings. In a recent report, 13 was shown to inhibit the in vitro peroxidation of LDL.[111]



13

Combination Therapy - The clinical use of two lipid regulators in combination has provided superior efficacy for the treatment of severe hypercholesterolemia. In a recent study, efficacy (LDL lowering) with the combination of probucol (1g/day) and colestipol (10g/day) was as great as with the full dose of resin (20g/day),[112] and without the gastrointestinal side effects associated with resin treatment. However, the resin did not prevent the decrease in HDL induced by probucol.[112,113] The combination of neomycin and nicotinic acid decreased LDL-C more than with neomycin alone.[55] Compactin[114] and mevinolin[115] were shown to enhance the LDL-lowering due to resin therapy, primarily by increasing the fractional clearance rate of LDL.[115,116] Such combinations produce reductions of LDL similar to those reported with colestipol plus nicotinic acid (50-55%)[117] although the mechanism for the latter is not as evident.[39]

Antihypertensive Drugs and Dyslipidemias - Certain classes of antihypertensive drugs have been found to affect TG and C metabolism and result in an undesirable plasma lipoprotein profile. Since antihypertensive therapy is long-term and such changes in lipoprotein metabolism may promote the atherogenic process, the situation arises whether high blood pressure is being controlled at the expense of increasing the effect of other major risk factors, hypercholesterolemia and/or hypertriglyceridemia. It is interesting that although antihypertensive drugs have been effective at decreasing mortality due to stroke and congestive heart failure, they have been relatively ineffective at protecting against CHD and MI.[118] Loop diuretics, potassium savers, thiazides, and related drugs have been shown to increase both total plasma TG and VLDL-TG values in studies ranging from three weeks to one year in duration.[119,120] In addition, serum C levels are increased (5-10%) with diuretics, the effect due predominantly to increases in LDL-C (4-20%) while HDL-C is relatively unaffected.[121,122] In contrast, peripheral and central sympathetic blocking agents such as reserpine and methyldopa depressed HDL-C levels, but did not adversely affect TG concentrations.[123]

Of the β-blockers, propanolol has been the most widely evaluated for effects on lipoprotein metabolism.[122-126] More dramatic increases in VLDL-TG levels were observed with both selective and nonselective β-blockers than those possessing intrinsic sympathomimetic activity (ISA). However, some studies showed a significant reduction (15%) in HDL-C levels without affecting total C and LDL-C values.[124,125] An added problem with propanolol is the propensity for elevated uric acid and glucose levels.[125] Thiazide diuretics can cause hyperglycemia and inhibition of LPL due to peripheral insulin resistance and cAMP-stimulated lipolysis in adipose tissue, which increases both hepatic and extrahepatic originated substrate for TG synthesis.[120] In contrast, β-blockers inhibit adenyl cyclase activity in adipose tissue leading to reduced hormone-sensitive lipase activity and decreased mobilization of fatty acids.[123] Recent evidence indicates that the plasma half-life of an intravenous TG emulsion is increased with β-blockers without ISA.[126] However, long-term studies with labetolol, an α- and β-adrenoceptor blocking drug, on plasma lipoproteins and their metabolism showed no adverse effects.[127]

In contrast to the effects of both thiazide diuretics and the β-blockers, the postsynaptic selective α_1-antagonist prazosin has not only been shown to be an effective antihypertensive agent, but also to produce a healthier lipoprotein profile. In both man and animals, prazosin reduced circulating plasma TG and VLDL-TG levels.[123-126,128] In chow-fed rats and rats fed a high glucose diet prazosin lowered plasma TG concentrations by 40-50% with the change being secondary to a fall in VLDL-TG secretion rate.[128] In studies of the effects of prazosin on human plasma and lipoprotein-lipid indices, beneficial alterations in HDL-C, LDL-C, and VLDL-C and total TG values were achieved. In addition, prazosin in combination with certain diuretics or β-blockers reversed the undesirable effects of these two classes of antihypertensive agents on plasma TG and VLDL-TG levels, and to a lesser extent on HDL-C and LDL-C.[129-131] Another α-antagonist, urapidil, was neutral in terms of lipid responses.[133]

Two other classes of antihypertensive agents, vasodilators and ACE inhibitors, have also been evaluated for effects on lipoprotein metabolism. The vasodilator carprazidil (RO 12-4713) consistently increased HDL-C without affecting other lipid and lipoprotein parameters during 4 months of therapy.[132] The ACE inhibitor captopril produced no consistent adverse alteration in plasma C and TG levels in both short- and long-term clinical trials.[134]

Importance of Calcium in Atherosclerosis - There is increasing evidence that ionic calcium (Ca^{++}) may play an important role in the pathogenesis of atherosclerosis. Under normal conditions, the influx of Ca^{++} into cardiovascular cells appears to serve as a "second messenger" and its binding to calmodulin intracellularly mediates numerous Ca^{++}-dependent functions, i.e., contraction-relaxation in contractile cells, chemotaxis, stimulus for mitosis, various platelet functions, secretion of connective tissue components, endocytosis of LDL and cellular energy production. However, the fatty streaks appearing in childhood, which contain calcified vesicles from degenerated cells of the intima and media, have provided evidence for the early onset of abnormal Ca^{++} metabolism.[135] Further analysis of these necrotic regions indicated a diffraction pattern similar to apatite and Ca^{++} bound to selected proteins. In late-stage plaque development, mineralization of connective tissue and cell debris have also been reported. In addition, elastinolysis and necrosis have

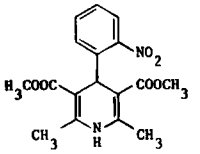
resulted from intracellular Ca^{++} accumulation causing cell death and release of lytic enzymes.[136,137] Extracellular Ca^{++} has been shown to mediate macrophage elastase activity, increase binding of lipoproteins to connective tissue proteins, and promote calcification of surrounding soft tissue.[138-141] Alteration in membrane lipid composition has also augmented Ca^{++} entry and has had deleterious effects on arterial cell function.[142,143] Constrictor effects in isolated canine coronary arteries by a high C environment were blocked by calcium antagonists.[136] Thus, these studies support the regulation of Ca^{++} entry into arterial cells as an approach toward beneficially affecting the atherosclerotic process.

Anti-Atherosclerotic Effects of Chelating Agents and Calcium Entry Blockers - Subcutaneous injections of the chelating agent magnesium ethylenediamine-tetracetic acid (EDTA) to C-fed rabbits exerted an anti-atherosclerotic effect by significantly reducing arterial elastin and collagen.[144] Recently, several other calcium chelating agents were tested in both New Zealand white rabbits and in monkeys (Macaca fascicularis) fed atherogenic diets.[141] The drugs used in these studies included ethane-1-hydroxy-1,1-diphosphonic acid (EHDP), azacycloheptane-2,2-diphosphonic acid (AHDP), 2-iminopyrrolidone-5,5-diphosphonic acid (IPDP), amino-1-hydroxy-propane-1,1 diphosphonic acid (APDP), lanthanum ($LaCl_3$), an inhibitor of calcium uptake via receptor channels and trimazosin, a cAMP phosphodiesterase inhibitor. Although not one of these compounds showed significant plasma lipid-regulating activity, all suppressed aortic accumulations of calcium, phosphorus, collagen and elastin, and reduced aortic accumulation of C and minimized foam cell formation and intimal thickening. More recent reports have confirmed the anti-atherosclerotic effect of EHDP in swine and extended the observations in rabbits to include a reduction in the amount of necrosis in early lesions.[145,146]

Since calcium entry blockers inhibit calcium influx without causing bone demineralization, nifedipine (14) has been studied in rabbits with diet-induced atherosclerosis.[147] Although changes in plasma lipids did not occur, significant reductions in sudanophilic lesions, aortic C content and aortic calcium were found compared to untreated controls. Similar results for both nifedipine and nicardipine (both at 40 mg/kg bid) were recently reported.[148] Low dose nifedipine (2 mg/day) was also effective in rabbits.[149] In Watanabe heritable hyperlipidemic (WHHL) rabbits, which lack the LDL receptor, nifedipine (40 mg/day over 26 weeks) showed no obvious difference in plaques covering either the aortic arch or the thoracic aorta when compared to an untreated group.[150] In another study, nifedipine (20 mg bid) was given to Japanese white rabbits fed a 2% cholesterol diet for 12 weeks compared to WHHL rabbits fed chow for 20 weeks. Atherosclerosis was suppressed in the Japanese rabbits but not in WHHL rabbits.[151] Other investigators have also failed to demonstrate an anti-atherosclerotic effect of nifedipine in C-fed rabbits.[152,153] In rhesus monkeys treated with nifedipine over 12 months and fed an atherogenic diet, no significant effect on any aspect of atherosclerosis was observed.[154]

Verapamil (15), a slow channel calcium antagonist, showed protective effects on atherosclerosis in C-fed rabbits,[155] but not in WHHL rabbits.[156] In a recent study using verapamil and a lipid-lowering diet in rabbits with aortic atherosclerosis, the combination halted further progression of the disease in those rabbits previously fed a C-diet.[157] Diltiazem, propanolol, reserpine, guanethidine and

flunarizine are other compounds affecting arterial calcium deposition which have been reported to beneficially affect the atherosclerotic process.[158,159]



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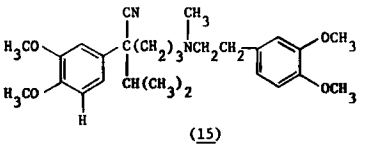

(15)

The mechanism of action of the chelating agents and calcium entry blockers at the cellular level is not well understood, but may be due to effects on smooth muscle cell migration, mitosis,[141] inhibition of collagen synthesis and secretion,[160] and/or a specific effect on cellular lipoprotein receptor activity. Similar mechanisms have been reported with diphosphonates.[164] Other proposed mechanisms include increased lysosomal and cytoplasmic cholesteryl ester hydrolase activity[165] and inhibition of ACAT activity via suppression of sterol carrier protein, both of which would reduce cellular C accumulation.[166]

Future Approaches - The complexity of communication between cells of the arterial wall and the disrupting effects caused by blood-borne cells (macrophages, platelets, neutrophils, etc.) interacting with them make the discovery of effective anti-atherosclerotic drugs a difficult task. Since cholesteryl ester accumulation in the atherosclerotic lesion is the hallmark of the disease, plasma lipid regulators have been given the most attention. Although substantiation of the LDL-C and HDL-C hypothesis is a step forward, minimal information presently exists about cellular events and interactions which are pro- or anti-atherosclerotic. As a better understanding of cellular processes and intercellular communications in the arterial wall is realized, more specialized therapy will become available. Some new approaches include anti-proliferative and anti-angiogenic effects of heparin-like molecules,[167-170] selective and nonselective inhibition of thromboxane and/or stimulation of prostacyclin formation,[174-174] regulation of specific cyclic nucleotides and phosphodiesterases,[175-178] and modulation of the release of cell secretory products affecting lipoprotein uptake.[179-182] Although these areas of investigation hold great promise, it is likely that no single form of therapy will prove efficacious for all patients at risk for premature CHD.

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Chapter 20. Therapeutic Approaches to Rheumatoid Arthritis and Other Autoimmune Diseases

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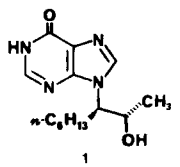
Introduction - The management of autoimmune disease, particularly rheumatoid arthritis (RA) and other related chronic, systemic and articular inflammatory diseases, is based upon current understanding of the pathogenic mechanisms involved.[1] The immune-based chronic inflammation, triggered by persistent bacterial, viral or autoantigens, or by an aberration in cytokine regulation of T-cells,[2] forms the underlying pathophysiology of RA and other autoimmune diseases, such as systemic lupus erythematosus (SLE), psoriasis and psoriatic arthritis, atopic dermatitis, ankylosing spondylitis (AS) and chronic inflammatory bowel disease. Current therapy for RA is divided into two main categories dependent on the severity of the disease. Symptomatic relief of the effects of acute inflammation is readily accomplished using non-steroidal anti-inflammatory drugs (NSAIDs), now well-established as the primary line of treatment.[3] In order to slow or halt tissue destruction, and more specifically joint damage, agents now classified as disease-modifying antirheumatic drugs (DMARDs),[4,5] such as gold, D-penicillamine (DPA) and the antimalarials, while not necessarily providing a cure, do retard the progress of the underlying disease in a less than predictable manner[6-8] by mechanisms as yet only poorly understood.[9,10] Since excellent reviews of the established NSAIDs[3,11] and DMARDs[12,13] have recently appeared, this report will focus on potential DMARD candidates operating by a variety of mechanisms, with brief updates on agents cited here previously.[14,15] Where appropriate, application of these and similar drugs to the treatment of other autoimmune disorders will be summarized.

Recent DMARD Candidates - The difficulties inherent in discovering new DMARDs for RA and other autoimmune diseases are two-fold. First, there exists no single biological model of RA which can consistently predict DMARD-type activity. Secondly, an extended clinical trial of a potential DMARD double-blind against placebo is considered unethical since a statistical number of RA patients, whose disease is severe enough to be otherwise treated, will go untreated for upwards of a year or more. Hence, most potential DMARDs are screened and clinically evaluated against the standard agents cited above. Alternatively, clinical agents of approved therapeutic utility in other autoimmune diseases are examined in the treatment of RA. A number of such crossover DMARD candidates are currently being evaluated. Sulfasalazine, useful in the treatment of ulcerative colitis,[16,17] has been evaluated against other DMARDs in a number of RA studies, which indicate that long-term sulfasalazine treatment of RA is a viable option, especially in patients resistant to gold or DPA.[18-20] A controlled double-blind study of etretinate, an oral retinoid used in the treatment of psoriasis, provided modest confirmation of previously uncontrolled studies in psoriatic arthritis.[21] 13-*cis*-Retinoic acid and N-4-hydroxyphenylretinamide (fenretinide) both significantly reduced rat adjuvant arthritis (rat AA).[22,23] The latter also suppressed streptococcal cell wall-induced arthritis.[24] Another DMARD candidate, thalidomide, previously used as a sedative, hypnotic and antiemetic until withdrawal because of its severe teratological side effects, showed therapeutic utility in a form of leprosy, discovered while a patient

received it as a sedative. Its anti-inflammatory properties in rat AA have prompted investigation into its DMARD potential for other autoimmune states.[25,26] In a small, open RA clinical study, oral administration of thalidomide led to marked clinical improvement, which, in some cases, lasted long after discontinuation of dosing. Adverse reactions, mainly associated with sedative effects, disappeared at the completion of dosing.[27] Although its extreme profile of side effects limits the potential use of thalidomide in RA, chemical modifications designed to retain the anti-inflammatory properties while eliminating adverse effects are ongoing.[28] Finally, the biological and pharmacological profile of clobuzarit (Clozic®), a compound originally taken into clinical trials for atherosclerosis but later found to possess distinct DMARD properties in RA, has been summarized.[29] A weak anti-inflammatory agent, clobuzarit inhibits the acute phase of rat AA, and may be acting by antiproliferative pathways. Adverse side effects observed in clinical trials have forced the withdrawal of clobuzarit from further consideration.[30]

Immunomodulatory Drug Therapy - Perturbation of the delicate balance in the cellular and humoral immunoregulatory network results in a net expression of aberrant hyperreactivity in most autoimmune diseases.[31] Thus, although immunosuppression might seem warranted for diseases of chronic inflammation and immunologic hyperreactivity, immunomodulation (immunoregulation), the dampening of hyperactive responses or enhancing defective negative influences, particularly through manipulation of the soluble mediators interleukins 1 and 2 (IL-1 and -2),[32] is the required therapeutic strategy.[33,34] Two immunomodulators discussed here previously have gained limited approval for use in RA and other autoimmune diseases. Levamisole was shown to be superior to placebo[35] and comparable to gold and DPA[36] in two large RA trials, and of possible use in Chron's disease, herpetic keratitis[37] and AS.[38] Although patient withdrawals for toxicity reasons were frequent with levamisole, the side effects were found to be most rapidly reversible. Variations in the immunomodulatory effect of thymopentin (TP-5) and other thymic hormone derivatives approved for use in thymic deficiency, have been found to be dependent on the mode of administration,[39] and there are conflicting reports on its clinical effectiveness in RA.[40,41] Hypoxanthine derivatives hydroerythranol (NPT-15392, 1) and isoprinosine (INPX, 2) were found to enhance T-cell lymphocyte differentiation and natural killer (NK) cell activity.[42-46] One of these studies,[45] carried out in patients with prolonged generalized lymphadenopathy, and other similar studies have prompted the investigation of INPX as a treatment in the early stages of acquired immune deficiency syndrome (AIDS). While INPX has been suggested as a treatment for RA[47] and for SLE,[48] no controlled trials have been carried out. In a small trial, however, INPX was found to be highly efficacious in the treatment of aphtous stomatitis, a result attributed to increased IL-2 production.[49] Lobenzarit (CCA, 3), previously reported to inhibit rat AA, was examined in vitro at non-toxic levels, and was found to block IL-1 and Ig production without effect on IL-2, and to induce γ-interferon (γ-IF).[50] The therapeutic effect observed is postulated to be mediated by short-lived, thymus-derived lymphocytes,[51] rather than macrophages.[52] In a double-blind study, CCA was significantly superior to placebo, although a number of side effects were noted.[53] The isoxazole HWA-486 (4), currently in clinical trials, prevents the onset of rat AA, provided therapy is initiated within the first 12 days



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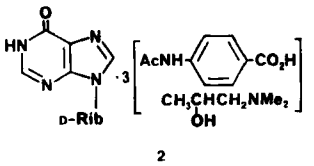

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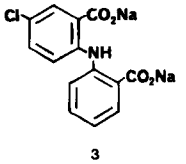

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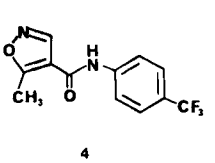

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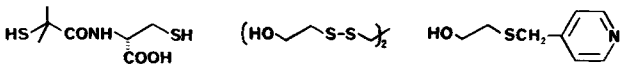
of induction. After this point, it behaves as a classic NSAID, reducing inflammation only as long as administered.[54] This response is correlated with improved lymphocyte response to both T- and B-cell mitogens and enhanced monocyte activity.[55] Thiol-containing compounds based on the lead provided by DPA continue to be investigated. Tiobutarit (SA-96, 5), currently in advanced trials, differed from DPA in various immunopharmacological models, was more potent than DPA as an inhibitor of macrophage migration,[56] and inhibited rat AA alone or in conjunction with indomethacin.[57] A summary of the immunological profile[58] and the results of a preliminary clinical trial[59] have appeared. In in vitro and in vivo studies sodium diethyldithiocarbamate (DTC, Immuthiol®) enhanced T-cell specific functions in mice, and, in certain instances, IL-2 production in human T-lymphocytes,[60] prompting limited studies in both AIDS[61] and chronically infected patients.[62] ADA 202-718 (6) activated lymphocytes both in vitro and in vivo, potentiating IL-2 and IF release in response to allogenic stimulation.[63,64] While it did not decrease swelling in rat AA, both pain and locally induced edema were reduced.[64] Ristianol (7) has been registered as an



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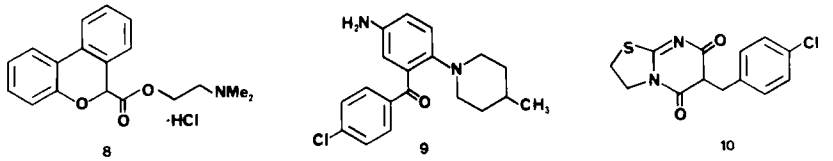
antiinflammatory immunoregulator in Europe, but no further information is available.[65] In a six-month open trial of the angiotensin-converting enzyme (ACE) inhibitor captopril in RA, the results compared favorably with similar DPA or gold studies.[66] A control study using enalapril, a non-sulfhydryl ACE inhibitor, was completely negative, suggesting that the observed antirheumatoid activity of captopril is due to the thiol group and not to ACE inhibition.[67]

Increased emphasis on immunomodulatory RA therapy has produced a number of new agents of widely varying structural types. FCE 20696 (8) exhibits antiviral activity mediated by IF induction,[68] and is orally active in three models of autoimmunity [rat AA, experimental allergic encephalomyelitis (EAE) and NZB/WF_1 mice].[69] LF-1695 (9), which potentiates suppressor T-cell mechanisms[70] and induces both IL-1 and -2 synthesis,[71,72] is being evaluated in



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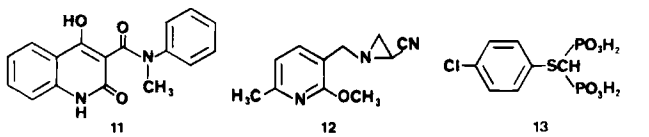
clinical trials.[73,74] TEI-3096 (10), shown to suppress rat AA without effects on acute inflammation by restoration of suppressor T-cell activity,[75] is currently in advanced clinical trials as an antirheumatic agent. Roquinimex (LS-2616, 11) augmented mouse NK cell activity but did not increase serum IF levels,[76] and enhanced responses to T-cell mitogens and the delayed-type hypersensitivity (DTH) reaction.[77] Lymphocytes from treated rats also showed enhanced IL-2 production, and restoration of immunosuppression characteristic of inhibition of IL-1 production. Ciamexon (BM 41.332, 12) produces significant reduction of symptoms in rat AA, but differed clearly from standard antirheumatic agents. Its effect was attributed to a selective stimulation of the maturation of T-cells, such that helper T-cells cannot be developed and the differentiation to suppressor



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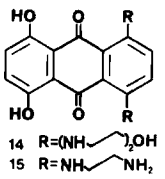
T-cells is increased.[78] An increase in DTH response[79] and in the survival rate of immunocompromised mice[80] have also been observed. Diphosphonate SR-41319 (13) is effective in the suppression of rat AA, likely by alteration of hydroxyadipate crystal disposition,[81] and inhibits activities characteristic of IL-1 stimulation.[82] The immunomodulatory activity of retinoids has been reviewed,[83] and is likely the mechanism of action of etretinate and other retinoids in the treatment of psoriatic arthritis mentioned above.

Immunosuppressive agents such as methotrexate (MTX), cyclophosphamide (CP) and azathioprine (AT) are limited in their use due to associated toxicities,[84] whereas non-cytotoxic agents offer another possibility in autoimmune therapy. Cyclosporin A (CS), an immunosuppressive agent approved for post-transplant use,[85] was highly efficacious as a potential DMARD in RA in two trials,[86,87] and has been proposed for use in psoriatic arthritis,[88] SLE and insulin-dependent diabetes.[89] The prospect of further modifications to CS to narrow its immunosuppressive profile has been proposed.[90] Other synthetic immunosuppressive agents have also been shown to be potential RA treatments. Mitoxantrone (14) inhibited helper T-cell function while enhancing suppressor activity,[91] and suppressed the EAE reaction in rats.[92] In rat AA, it displayed potency and efficacy comparable to MTX and greater than CP.[93] AQAD (CL-232,468, 15), a derivative of mitoxantrone, is considerably more potent, inhibiting in vitro induction of alloreactivity and impairing both the proliferative response of lymphocytes to antigen and the generation of cytolytic T-cells. In vivo, the suppression persisted for as long as 30 days after a single i.p. dose, consistent with the induction of suppressor T-cells as the mechanism of action.[94] CL-246,738 (16) possesses a broad in vitro and in vivo immunomodulatory spectrum, including activation of NK cells and macrophages, and induction of IF.[95,96] A structurally unrelated compound from the same group, CL-259,763 (17), stimulated macrophages and cytolytic T-cells.[97] Sch-24,937 (18) suppressed the secondary response in rat AA, and was more potent than AT but less potent than CP in EAE. In contrast to many other immunosuppressive agents, it did not cause a reduction in circulating lymphocytes, indicating that its mode of action is not cytotoxic.[98]



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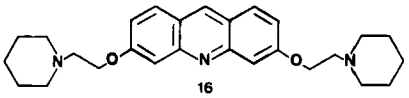

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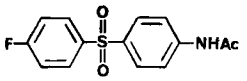

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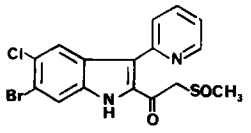

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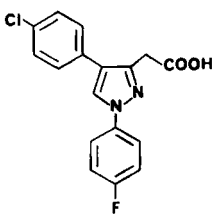
NSAIDs as Immunomodulatory DMARDs - Although NSAIDs are thought to operate by a common mechanism of action, observed interpatient differences in drug efficacy have raised questions about additional modes of anti-inflammatory action independent of prostaglandin (PG) synthetase inhibition.[99] Since arachidonic acid metabolites are known to exert a range of immunoregulatory activities,[100] the examination of the interrelationship of NSAIDs and other mediators of inflammation present in RA revealed a link between these agents and both humoral and cellular immune response. NSAID inhibition of the production of PGE_2, the PG shown to tonically inhibit suppressor T-cell activity, allows suppressor T-cells to mature and control B-cell mediated rheumatoid factor production.[101,102] In in vitro and in vivo studies, NSAIDs inhibited a number of markers of neutrophil activation, such as migration, superoxide generation and aggregation.[103-105] The patterns of this inhibition vary from drug to drug, suggesting that NSAIDs may directly effect neutrophil activation by pathways independent of their shared inhibition of PG synthesis, such as their capacity to inhibit movements of calcium and enhance intracellular cyclic AMP

(cAMP) levels.[106]

The combination of NSAID and DMARD profiles has been suggested as the ideal drug treatment for RA. A number of NSAIDs either established or under investigation have been found to display this profile. Of the acidic drugs, fenclofenac,[107] diclofenac sodium[108] and the new agents pirazolac (MY-309, 19)[109] and RS-2131 (20)[110] display evidence of modest immunomodulatory activity. Wy-18,251 (21), originally identified as an antimetastatic agent with immunomodulatory activity,[111] is similar to levamisole both in vitro and in vivo.[112] In acute anti-inflammatory studies, 21 demonstrated activity comparable to aspirin, but without antipyretic activity, and also inhibited rat AA. In in vitro studies, it was a modest cyclooxygenase (CO) inhibitor lacking lipoxygenase (LO) inhibitory activity.[113] Wy-41,770 (22), an anti-inflammatory agent devoid of GI side effects, was active in rat AA, but was a weak inhibitor of PG synthetase activity.[114] In type II collagen-induced arthritis, 22 was more potent than indomethacin, methylprednisolone or DPA in reducing both hind paw edema and bone pathology.[115,116]



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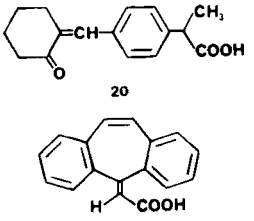

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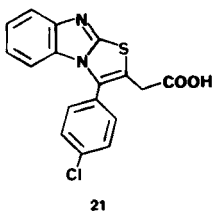

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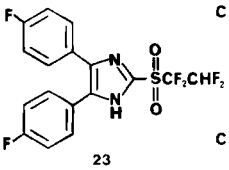
Imidazole- and thiazole-containing compounds constitute another class of NSAIDs which possess immunomodulatory activity. Tiflamizole (23),[117] structurally related to the platelet aggregation inhibitors flumizole[118] and fenflumizole,[119] is a potent CO inhibitor which inhibits rat AA,[120] and displays immunomodulatory activity in models of lymphocyte blast transformation and SLE.[121,122] Imidazothiazole 24, a hybrid structure derived from flumizole and levamisole, is an inhibitor of both phases of rat AA, and is active in a number of immunoregulatory assays.[123] Lotifazole (F-1686, 25)[124] and fanetizole (CP-48810, 26),[125] both weak CO inhibitors inactive in rat AA, display properties of immunostimulants in other assays. Lotifazole reduces DTH-induced pleurisy



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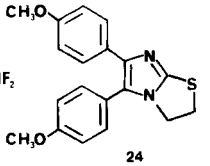

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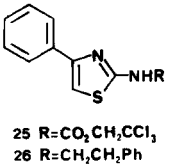
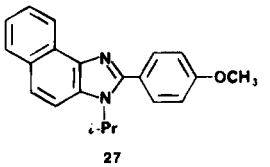


25 R=CO2CH2CCl3
26 R=CH2CH2Ph



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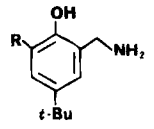
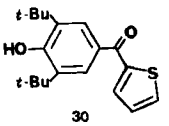
in guinea pigs and oxazolone-induced contact hypersensitivity in mice. Fanetizole specifically suppressed de novo IgE synthesis in atopic subjects. Both compounds show a stimulatory effect on T-lymphocytes. Another member of this chemical class, tomoxiprole (MDL-035, 27), is active in rat AA and a potent PG synthetase inhibitor, but as yet no indication of immunomodulatory activity has been reported.[126,127]

Antioxidants and Peroxygenase Inhibitors - Arachidonic acid peroxidation products and reactive oxygen species, both responsible for inflammation and tissue damage, can be inhibited by a wide range of structural types. In addition to the nutritional antioxidants such as vitamins A and E, selenium and eicosapentaenoic acid,[128,129] many anti-inflammatory agents such as phenylbutazone, phenidone[130] and CCA[131] also act as general antioxidants. Interference with polymorphonuclear leukocyte (PMN)-dependent oxygen

intermediate generation has been suggested as the mechanism of action of thalidomide in RA.[132] Four phenols recently prepared as antioxidants also exhibit marked anti-inflammatory activity.[130] MK-447 (28) enhanced PGE_2 synthesis, but exhibited marked antihypertensive and diuretic effects in man.[133,134] ONO-3144 (29) acts as a general free radical scavenger and weak inhibitor of thromboxane synthetase.[135] In clinical trials, 29 has been well tolerated, providing improvement in RA and lumbago with only minor GI side effects.[136]


28 R=I
29 R=COEt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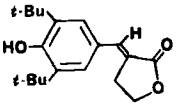
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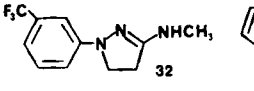
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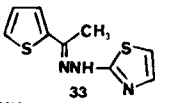
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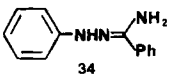
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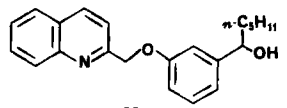
R-830 (30), currently in clinical trials for topical treatment of inflammatory dermatoses,[130] is orally active in rat AA, reversed passive Arthus reaction, and oxazolone-induced DTH, and inhibits both CO and LO.[137] Reports on KME-4 (31) show a similar dual inhibitor profile of anti-inflammatory activity.[138,139] The combination of the anti-inflammatory effect of CO inhibition with the potential immunomodulatory effects[140] of LO inhibition has been proposed as a new treatment for RA.[141,142] BW-540C (32) is active against carrageenan-induced edema in rats, and has been selected for evaluation in the treatment of psoriasis.[143] CBS-1108 (33)[144] and CBS-1114 (34)[145] both exhibit a wide range of anti-inflammatory activities, especially in models in which CO inhibitors are not effective. The former exhibited a marked effect on leukocyte chemotaxis.[146]

The products of 5-LO, particularly leukotriene B_4 (LTB_4), are potent chemotactic agents which elicit a variety of acute[147] and immune-based[138] inflammatory responses. Both REV-5901 (35)[148,149] and RS-43179 (36)[150-152] were potent and selective 5-LO inhibitors both in vitro and in vivo in the topical arachidonic acid-induced mouse ear edema model.[153-155] In clinical trials, 36 was effective as a topical treatment for psoriasis.[156] The lipophilic benzoquinone AA-861 (37), also a selective 5-LO inhibitor, exhibited effects on asthmatic and inflammatory reactions.[157] Although the reported LO inhibition by sulfasalazine has been disputed,[158,159] one primary metabolite, 5-aminosalicylic acid, inhibits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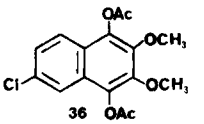
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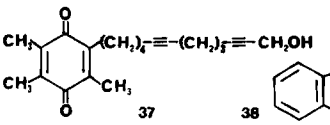
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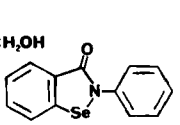
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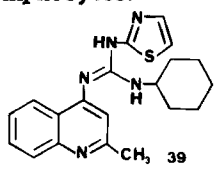
5-LO[160] and is thought to contribute to the first line effect in RA, while the other metabolite, sulphapyridine, is the active moiety responsible for second line effects.[161] Both prodrug[162] and controlled delivery[163,164] systems for 5-aminosalicylic acid release in ulcerative colitis treatment have been described. Although benoxaprofen has been demonstrated to be a LO inhibitor of some effect in psoriasis,[165] a small trial in ulcerative colitis proved unencouraging.[166] Regulation of glutathione peroxidase, responsible for the decomposition of hydrogen peroxide into toxic oxygen species, was proposed as the mechanism of action for the novel organoselenium compound ebselen (PZ-51, 38),[167] but a more recent report indicates that 5-LO inhibition is the probable mechanism of action.[168] Ancillary to the direct inhibition of 5-LO, the potential utility of antagonists of LTB_4[169] and platelet activating factor[170,171] in inflammation has been discussed.

Phospholipase and Phosphodiesterase Inhibitors - Glucocorticoids are known to exert their anti-inflammatory action by inhibition of the release of arachidonic acid from phospholipids by phospholipase A_2 (PLA_2).[172] Detection of PLA_2 in the sera and synovial fluids in RA[173] reinforced the search for non-steroidal PLA_2 inhibitors for chronic inflammation, even though the list of known inhibitors is short[174] and recent detailed inhibition studies have raised questions concerning PLA_2 activity.[175] Timegadine (39), shown previously to inhibit rat AA, may exert its dual CO-LO inhibition by inhibition of PLA_2.[176] A small clinical trial has established its potential effectiveness in the remissive control of RA.[177] Glucocorticoids inhibit PLA_2 by stimulation of a 15 kilodalton cell membrane protein variously referred to as macrocortin, lipomodulin or (the now preferred) lipocortin (LC), the endogenous inhibitor of PLA_2.[178] Highly purified LC exhibits steroid-like effects and an acute anti-inflammatory activity in rat carrageenan pleurisy edema.[179,180] Monoclonal antibodies to LC reversed the biological effects of steroids in vitro and to a certain extent in vivo.[181,182]

Cyclic nucleotides (cAMP or cGMP) are known to affect a multitude of biochemical regulatory processes, including the immune system.[183] Inhibition of the phosphodiesterases (PDEs) present in inflammatory cells would consequently raise intracellular levels of cAMP or cGMP, which in turn influence many cellular processes responsible for inflammation, including chemotaxis, aggregation and release of lysosomal enzymes and inflammatory mediators. Recently, nitraquazone (TVX-2706, 40) has been found to be a selective non-competitive inhibitor of cAMP PDE from a variety of cell sources, including PMN,[184] possibly accounting for its potent anti-inflammatory activity.[185] The observed immunomodulatory activity of 1 may be due to selective inhibition of lymphocyte cGMP PDE.[186] In vivo administration of CS was also found to raise cAMP levels in rat lymphocytes.[187]



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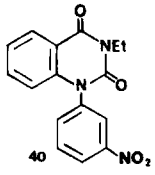

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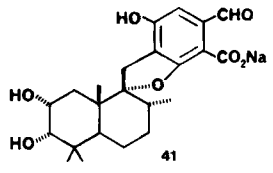

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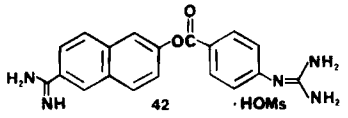

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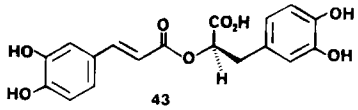

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Inhibition of Complement - The normally beneficial effects of the complement cascade, an immunostimulated process, lead to the adverse symptoms of chronic inflammation in autoimmune disease. Inhibition of the complement alternative pathway (C3 transformation) or the terminal complement reaction (C5a activation) has been proposed as a possible treatment for RA, SLE, atopic dermatitis, psoriasis and other immune complex diseases.[188,189] The sesquiterpene K-76COONa (41) interferes with C5a activation, and inhibits leukocyte migration and release of histamine in inflammatory models.[190,191] Nafamstat mesilate (FUT-175, 42), a synthetic protease inhibitor, inhibits the alternative complement pathway,[192] and has both prophylactic and curative effects on the development of lupus nephritis in mice.[193] Rosmarinic acid (43), a natural product exhibiting weak effects on mediators of acute inflammation, inhibits many inflammatory responses associated with complement activation, and is under development as a topical treatment for various autoimmune-based skin disorders.[194]

Conclusion - Recent research has allowed a better definition of the basis of autoimmune disease, even though the precise details of the pathogenesis and etiology of most of these disorders remain unknown. Advances in immunology, in

particular the complexity of the requirements for self-recognition, have shown that this internal regulatory system is subject to a myriad of controlling mechanisms and multiple pathways by which these mechanisms may be rendered abnormal.[1] Pharmacological manipulation of even one of these regulatory systems might provide the means to control and eventually reverse the abnormal anti-self immune responses characteristic of autoimmune disease.

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Section V - Topics in Biology
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SPECIAL TOPIC

Chapter 21: The Receptor: From Concept to Function

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Introduction - The working concept of receptors has undergone a continuous evolution since the turn of this century. Observations that neurotransmitter, drug and hormone effects vary with concentration and chemical structure, are stereoselective, and may be pharmacologically antagonized or reversed, have all pointed toward the existence of distinct cellular constituents that are capable of recognizing subtle differences in the chemical composition of ligands. Since its conceptualization, the receptor has formed the cornerstone of pharmacology.[1,2] Thus for over seventy years medicinal chemists have been concerned with determining the optimal structure-activity relationships (SAR) of compounds as they relate to something which has been more of a concept than a tangible entity. As advances in molecular biology have allowed the isolation, cloning and sequencing[3,4] of receptors, which in the case of the nicotinic cholinergic receptor[5] includes a complete knowledge of its primary structure, the assumption has been made that receptors are now readily amenable to molecular characterization. However, receptors are still typically characterized in terms of function rather than their structural properties.

From a biochemical standpoint, receptors are typically treated as enzymes, both in terms of function and kinetics.[6,7] However, unlike enzymes, the receptor substrate, the agonist, is normally not altered as part of the reaction sequence. Thus the consequence of a receptor-ligand (RL) interaction is not a by-product of the ligand but rather a change in the intracellular milieu (cyclic nucleotide formation, alteration in ion flux or phospholipid turnover). Furthermore, the events related to receptor activation are sufficiently complex that its removal from the membrane microenvironment can result in the loss of components that are essential for function. Whereas low molecular weight cofactors (i.e. NAD, Mg^{++}) necessary for enzyme activity can be added to an isolated preparation, this may not be possible for receptors inasmuch as their function may require an association with membrane constituents that are molecularly distinct from the recognition site.

The term 'receptor' may refer to several distinct molecular entities. While receptors were originally conceived as the recognition sites for drugs and hormones, this nomenclature now often includes those associated components necessary for function (effector mechanism). In the present review, 'receptor' refers only to the membrane recognition site. It is important to note that receptors, by definition, are functional entities, whereas drug recognition or binding sites are not always associated with a functional component of the cell. The chemist concerned with drug synthesis is primarily interested in whether or not a particular agent has activity at a given receptor, whether such activity is sufficiently selective to be of therapeutic value with limited side effects, and whether the substance will be active in the intact animal. Selectivity (a recognition site phenomenon) and activity (a recognition site/effector function) are well defined pharmacological phenomena, whereas activity following systemic administration is dependent upon pharmacokinetics and bioavailability which have yet to reveal stringent structure-activity relationships.

The present review is designed to highlight developments in receptor classification and characterization as they relate to drug action and the design of new chemical entities. While emphasis is placed on neurotransmitter receptors, the basic principles apply to any type of RL interaction. Other concepts, such as receptor modulators, endogenous ligands, and drug receptors, are also discussed.

Receptor Theory - Many of the theoretical approaches used for describing RL interactions, while intellectually appealing, appear at times to be only remotely related to the characterization of drugs as therapeutic agents. The simplest model, the occupancy theory of Clark, is the best appreciated, and its practical ramification, the saturation isotherm, the most widely used method for characterizing receptors.[8]

Occupancy theory, like most receptor models, is based on the following assumptions:

1. the receptor-ligand interaction is reversible,
2. association is a bimolecular, and dissociation a monomolecular process,
3. all receptors of a given class are equivalent and independent of one another,
4. receptor occupancy does not alter 'free' receptor affinity or the free ligand concentration, and
5. the biological response elicited by receptor activation is directly proportional to the number of occupied receptors,
6. the biological response is dependent on the attainment of an equilibrium between the receptor and its ligand.

7. The receptor, in its role as a transducing element, does not modify the ligand but is itself shifted from an inactive to an active state.

The saturation isotherm is equivalent to the Langmuir absorption isotherm and, at equilibrium, the kinetics of the RL interaction may be described in terms of the Hendersson-Hasselbach equation. In practical terms these reduce to the Michaelis-Menten equation that may be plotted in a variety of ways as described by Scatchard,[9] Eadie-Hofstee,[10] Rosenthal,[11] and Woolf[12] to derive an affinity constant (Kd) and a binding site density (Bmax).[13] When receptors are characterized by ligand binding assays, the saturation isotherm is generated by incubating a fixed concentration of tissue with increasing concentrations of the radioligand in the presence and absence of a saturating concentration of unlabeled ligand. The amount of radioligand bound to the tissue in the presence of the unlabeled species (the unsaturable component) is subtracted from that bound in its absence (total binding) to calculate the amount of radioligand attaching to the receptor site (displaceable or specific binding). Under equilibrium conditions, the amount of specific binding increases as a function of the radioligand concentration over a limited range, beyond which specific binding remains constant (saturation). The slope of the saturation curve reflects the affinity of the receptor for the radioligand, whereas the point of inflection indicates the density of binding sites. Assuming a uniform population of receptors, the concentration of radioligand yielding a half-maximal saturation is equivalent to the Kd. In transforming saturation isotherms to the Scatchard plot, the amount of ligand bound (B) is plotted on the abcissa against bound/free (B/F) on the ordinate. The y intercept is Bmax/kd and the x intercept, Bmax.

An alternative theory is that proposed by Paton where activation is proportional, not to the number of occupied sites, but rather to the frequency of occupations per unit time.[14] While this allows for a kinetic discrepancy between binding and effect under some situations, at equilibrium the occupancy and rate theories are mathematically equivalent.

More sophisticated models of RL interactions are continually being proposed, usually in an attempt to explain experimental data that deviate from mass action kinetics. Two models that have stood the test of time are the concerted model[15] and the sequential or induced fit model.[16] The strengths of these models are that they explain cooperative interactions among component subunits, or protomers, of an oligomeric receptor complex (Table 1).

While these models have been used extensively in enzymology, they have limited utility for explaining receptor sites. Because all current methods used for describing RL associations, either *in vitro* or *in vivo*, assume no interaction between receptors, it is doubtful whether events related to a receptor complex activation can be accurately characterized.[17] Exceptions to this are the models used

to describe agonist interactions with the nicotinic cholinergic receptor.[18] While many investigators are attempting to define receptors on the basis of a ternary complex that would permit analysis by the cooperative models, it is debatable whether current laboratory techniques are capable of distinguishing between artifacts and biologically meaningful data in this regard.

Table 1: Cooperative Models of Receptor Action

Concerted Model (Monod et al)	Sequential Model (Koshland et al)
The receptor is a multicomponent oligomer comprised of a finite number of identical protomeric subunits symmetrically arranged, each with a single binding site for the ligand.	The receptor is a multicomponent oligomer. Protomers, each with a single ligand binding site, are arranged symmetrically.
The receptor exists in two conformational states, one of which has preferential affinity for the ligand.	Protomers can exist in two conformational states, transition between which is induced by binding of the ligand.
Transition between conformational states involves a simultaneous shift in state of all protomers. No hybrids exist. Implies cooperativity.	Receptor symmetry is lost when the ligand binds. Hybrid states of the oligomer are stabilized by protomers. Hybrid stabilization is equivalent to negative cooperativity.

The occupancy theory[8] has undergone modification in the fifty years since its proposal, resulting in the intrinsic activity concept of Ariens (the Clark-Ariens formulation) and the receptor reserve or spare receptor theory of Stephenson.[20] The Clark-Ariens formulation proposes that the biological effectiveness of an agonist is determined, not only by its affinity for a receptor, but also by its ability to initiate a biological response. Thus, for a given series of agonists, the maximal response is proportional to both the agonist affinity and intrinsic efficacy. This concept, while initially supported by data indicating that the maximal response to a series of compounds can vary, is also illustrated when comparing binding affinities with biological responses. A pertinent example is the serotonin-1 agonist MK 212,[21,22] where the discrepancy between binding activity (weak) and biological response (strong) has been cogently related to efficacy.[23]

The receptor reserve concept was proposed when it was noted that some agonists produce a maximal effect in tissues even after a portion of their receptors had been irreversibly blocked.[20,24] This proposal differs from the classical notion of response being proportional to occupancy (Clark-Ariens) by suggesting that receptor saturation is not always necessary for maximal activity, and that the magnitude of the effect is related to an unknown function that lacks proportionality to receptor occupancy, although some relationship between response and occupancy does exist. In addition to introducing the idea of spare receptors, the reserve receptor concept accomodated the notion of full and partial agonists. Thus, a full agonist with high intrinsic activity may need occupy only a fraction of the available receptors to produce a maximal response, while a partial agonist (low intrinsic activity) may be incapable of eliciting a maximal response even with full receptor occupancy. Agonists that require full occupancy to elicit a maximal response are not included in Stephenson's theory.[24] The receptor reserve concept is also complicated by the existence of 'accessory receptors' that may be uncoupled from the effector system.[25] Functionally, the reserve receptor concept may be explicable in terms of a coupling between the receptor and its associated second messenger system, with some ligand binding sites being fully coupled while others are unaffiliated at certain times. Membrane fluidity may also allow for the existence of 'mobile' receptors, which when activated can produce a response that is not proportional to their occupancy.[26]

Receptor antagonists are, by definition, substances that prevent the expression of the biological response to agonists but are, by themselves, inactive. Antagonists are classified as competitive and non-competitive. The former interact directly at the agonist recognition site, while the latter reduce agonist activity by attaching to a separate site that allosterically modifies the receptor response. An example of a non-competitive antagonist is adenosine which, by activating the cardiac A-1 receptor and reducing the available pool of cyclic AMP, blocks the effects of β-adrenoceptor activation.[27] In general, competitive antagonists reduce the maximal response. Intermediate-type antagonists exist which modify both affinity and efficacy, as do functional antagonists which attenuate their own biological effects via distinct mechanisms.[28] Radioligand binding experiments have recently revealed the existence of compounds termed inverse agonists. These induce a receptor response that is opposite to that of a classical agonist. Thus, inverse agonists may be thought of as antagonists with reversed efficacy, while simple antagonists are agents with affinity but no efficacy. Examples of inverse agonists have been described for the benzodiazepine receptor[29,30] and include CGS 8216.[31] Other studies with this receptor complex have identified compounds such as etazolate[32] that enhance ligand binding. Analysis of such compounds has led to the realization that these actions may be related to competitive effects on endogenous ligand binding.[33] In the context of the inverse agonist nomenclature, such compounds could be termed 'inverse antagonists'.

Receptor Localization - In addition to being found on the cell bodies and dendrites of neurons representing the 'downstream' focus of a chemical pathway, receptors are also known to be present on the presynaptic terminal of cells containing their ligand. These autoreceptors are involved in feedback regulations of transmitter release.[34,35] In other cases, receptors may be located presynaptically on terminals of cells containing other neurotransmitters and in this way function to heterosynaptically affect the modulation of transmitter availability. Receptors for the transmitter released from a given neuron may also be located on its cell body,[35] playing a role in the overall integration of incoming signals. Other receptors, such as those mediating the action of steroids, directly convey information from the cell surface to the nucleus.[36]

Receptors In The Working Environment - In receptor theory there are certain key descriptors used to define RL interactions.

(i) The dissociation constant (KD) is derived from a saturation isotherm that is obtained by measuring the biological response as a function of the ligand concentration. Radioligand binding data can be transformed using the Scatchard[9] rearrangement to derive the Kd and an estimate of the Bmax.[13] Early workers obtained these values using simple graphical analysis, an approach fraught with problems due to the subjectivity of the method. Moreover, distortions occur as a result of the data transformation to a Scatchard plot which includes a variable (amount of ligand bound) on both the ordinate and abscissa, making it inappropriate to calculate the results by linear regression. These difficulties are overcome by analyzing the results using weighted nonlinear iterative curve-fitting programs such as LIGAND,[37] EBDA[38] and Lundon-1.[39] Such programs simplify the data analysis and are more rigorous. Caution must be exercised with this approach, since overinterpretation of computerized data may obscure the physiological significance of the findings.

(ii) The Bmax -Quantification of receptor density can yield important information with regard to receptor function. Thus, changes in the Bmax can be used as measure of drug efficacy, as has been the case for antidepressants.[40,41] Also, especially in regard to binding assays, the Bmax can be used to assess the physiological relevance of a binding site since under equilibrium conditions the apparent Bmax for most receptors is between 100-1000 fmoles/mg protein. Some may have densities greater than this (2-3 pmoles/mg protein), although values of 10 pmoles or greater suggest a nonselective site.

(iii) EC_{50} and IC_{50} Values - By comparing the effects of a given series of compounds it may be possible to establish an SAR for a receptor system. Agonist responses are expressed in terms of their concentration required to produce an effect equivalent to 50% of that seen with a reference agent (EC_{50}). Care must be taken in interpreting this value since the test compound may be a partial agonist, making it inappropriate to calculate its EC_{50} with respect to a full agonist. For antagonists, the equivalent measure is the IC_{50}. In another type of analysis an agonist is analyzed at various concentrations or doses in the presence of one of a number of antagonist concentrations to minimize the inaccuracies associated with the slope of a standard dose-response curve. Such data may be used for the calculation of the dose ratio (DR), a measure of the concentration of agonist necessary to overcome a given concentration of antagonist. A Schild plot is generated by comparing the log (DR-1) against the log of the antagonist concentration, yielding a pA_x value where A is the agonist concentration and x the DR.[42] The pA_x, usually presented as the pA_2, is an empirical measure of an agonist/antagonist interaction. The slope of the Schild plot is used to determine the applicability of a single receptor model and to characterize the interaction as competitive or non-competitive. A slope significantly below unity indicates that the interaction is non-competitive.

For radioligand binding assays, both agonists and antagonists yield IC_{50} values since the measure of activity is inhibition of radioligand binding. Interestingly, agonists as a group are generally more potent than antagonists in competing for the binding site if an agonist radioligand is used, whereas the reverse is true for an antagonist radioligand.[22] The IC_{50} values vary with the radioligand concentration. For instance, in early studies with the muscarinic cholinergic receptor ligand quinuclidinyl benzilate (QNB), its low specific activity required that a concentration be used that was many times higher than its Kd (60 pM).[43] Subsequent studies, using a ^{3}H-QNB preparation having a specific activity that allowed for the analysis of very low ligand concentrations, revealed that the IC_{50} values calculated for a series of unlabeled ligands differed from those obtained earlier since the unlabeled species had to compete with less ^{3}H-QNB to displace 50% of the radioligand. Inasmuch as each radioligand binding assay has its own set of experimental parameters, it is difficult to compare data between different assays. This problem is overcome by calculating an inhibition constant (Ki) for the test compound since this value is independent of the radioligand concentration. The Ki is calculated using the Cheng-Prusoff equation:[44]

$$Ki = IC_{50}/(1 + C/Kd)$$

where C is the concentration of radioligand used in the assay and Kd the dissociation constant for the radioligand. Thus, Ki values may be considered an absolute determination of the affinity of a substance for a given receptor, whereas the IC_{50} is relative to a particular set of assay conditions.

The absolute nature of the Ki is, however, dependent upon the compound interacting with only a single receptor subtype.

(iv) The Hill Coefficient - A measure of the potential existence of cooperativity in RL interactions is the Hill coefficient.[45] If the slope of the Hill coefficient is not significantly different from unity, then the degree of interaction between receptor sites is negligible. A Hill slope less than unity is suggestive of a non-competitive interaction between the ligand and its receptor, while a value greater than unity indicates positive cooperativity. In practice, deviations from unity for the Hill coefficient are usually interpreted in terms of receptor subtypes. It must be remembered that not all binding sites are receptors, and that the receptor designation can only be used when there is a correlation between binding and a functional response.[46,47] Thus, many 'lower' affinity binding sites that abound in the literature may not in fact represent a physiological receptor.

Receptor Sensitization - Receptor responsiveness can be described in terms of three concepts; subsensitivity, supersensitivity and desensitization. Supersensitivity occurs when the agonist tone has been reduced for a prolonged period and is reflected by a decrease in the concentration of agonist required to elicit a given response. Conversely, subsensitivity develops as a consequence of excessive agonist activity, making it necessary to increase the concentration of agonist to elicit a given response. Desensitization has a genesis similar to subsensitivity except that the maximal response is attenuated even at saturating concentrations of agonist. Desensitization in response to an agonist acting directly upon the receptor recognition site is termed homologous, whereas that resulting from an action at a different receptor that may be coupled through a common effector is termed heterologous. Changes in receptor number may occur by way of clustering, internalization, by an alteration in the synthesis or degredation of receptors, or by changes in their coupling to an effector mechanism.[48,59,119]

Modifications in receptor sensitivity may be an important regulatory mechanism for maintaining homeostasis and could be an underlying pathology in certain disorders.[51,52] Moreover, the clinical response to certain drugs, such as antipsychotics and antidepressants, may be related to their ability to alter the sensitivity of certain neurotransmitter receptors in brain.[53] Alterations in receptor affinity may also occur during drug administration. For example, it has been reported that treatment with diazepam induces a significant increase in benzodiazepine binding sites within an hour of administration.[54] Thus, as opposed to drug-induced changes in receptor number, which usually require several days or weeks of continuous treatment, modifications in affinity can occur relatively rapidly. Because affinity changes may be readily reversible, this phenomenon is more difficult to measure experimentally.

Recent studies have shown that protein phosphorylation catalyzed by cyclic AMP-dependent protein kinases may be one of the ways in which this second messenger alters receptor sensitivity and number.[55] Experiments with protein synthesis inhibitors have shown that receptors have half-lives on the order of several hours to a few days.[56,57] Such data suggest that receptors are not static entities, but rather should be viewed as dynamic constituents of the cell. Given these factors, it is conceivable that drugs binding irreversibly to a receptor complex could be therapeutically useful.

Receptor Classification - The identification of receptors and their subtypes depends entirely upon the availability of selective agonists and antagonists. Antagonists are particularly important since they provide the most convincing evidence that a response is receptor-mediated. Because endogenous substances are invariably agonists, the discovery of antagonists often occurs after a receptor subtype has been proposed.

Detailed on Table 2 are a number of receptors and their subtypes based on differences in ligand selectivity. It must be borne in mind that no compound can be considered entirely specific for a given receptor, which explains why some agents are listed under more than one putative receptor site. Nevertheless, the listed substances have been proposed to have some selectivity for the designated receptor, making them useful for defining these sites. The major gaps in this table relate to the absence of antagonists for many systems, including the $5HT_{1C}$, $GABA_B$, and most of the peptide receptors. Drug development in these areas would seem to hold great promise, not only from a therapeutic standpoint, but also for characterizing the biological significance of these sites.

Receptor-Effector Systems - Effector systems are crucial for the elaboration of the RL interaction, but are also the chief cause for the deviation between classical occupancy theory and experimentally derived data. The stoichiometry of the coupling between receptors and their effectors is not well-defined but, as discussed above, may account for the data suggesting the receptor reserve concept.

(i) Calcium is the oldest of second messengers, having been shown in the late-19th century to be essential for tissue contractility.[58] Calcium acts as a stimulus transducer by virtue of the extremely large (10,000-fold) concentration gradient that exists between the extracellular space and the cytosol.[59] This gradient is maintained by the low permeability of the plasma membrane to the cation, by active calcium exchange processes, and by intracellular sequestration. The cation is known to be intimately involved in processes related to stimulus-secretion coupling, a key event in converting electrically conducted nerve signals into a chemical event at the synapse.[60,61] Increases in intracellular calcium resulting from depolarization or by the mobilization of intracellular stores can initiate a number of biochemical and physiological processes (i.e. phosphorylation, muscle contraction). The importance of

Table 2: Receptor Classification and Associated Ligands

Binding Site or Receptor	Subtype	Agonists	Antagonists
ADRENERGIC			
	α_1[72]	Phenylephrine	*Prazosin[221]
		SKF 89748	*HEAT[120]
		St 587	*WB 4101[121]
	α_2[72]	*Paraminoclonidine[122]	*Rauwoulscine[125]
		BHT 920	*Idoxazan[126]
		*UK 14304[123]	*Yohimbine[211]
		*Clonidine[124]	
		Epinephrine	
	β_1[127]	Tazolol	*Carazol[220]
		Dobutamine	Atenolol
		Xamoterol	Practolol
		*Isoproterenol	Metoprolol
		HBI[130]	Betaxolol
		*CGP 12177[128]	*DHA[129]
		RO 363[225]	*ICYP[139]
	β_2[127]	Salbutamol	*DHA[129]
		Terbutaline	Butoxamine
		Zinterol	ICI 118,551
		Soterenol	Alprenolol[132]
		*HBI[130]	
SEROTONERGIC			
	5-HT_1[133]		
	A	*8-OHDPAT[134]	LY 165163[213]
		*PAPP[135]	
		*TVXQ 7821[136]	
		*WB 4101[212]	
	B	RU 24969	*ICYP[137]
		TFMPP[213]	
		CGS 12066B[228]	
	C	*Mesulergine[138]	
	5-HT_2	DOM	*Ketanserin[229]
		*DOB[139]	Pirenperone[141]
		MIL[140]	*Ritanserin
			LY 53857
			Xylamidine[142]
			*Spiperone
DOPAMINERGIC			
	D-1	SKF 38393	*SCH 23390[161]
		*ADTN[160]	*Flupenthixol[162]
			Pifluthixol[164]
	D-2	*Apomorphine[163]	*Sulpiride[165]
		LY 171555	*Spiperone
		NPA[230]	
	Autoreceptor	CGS 15855A[96]	
		RDS 127	
		3-PPP	
	DA-1	Dopamine	*(R)Sulpiride[166]
			*SCH 23390
	DA-2	Apomorphine	(S)Sulpiride[166]
		ADTN	

Table 2: Receptor Classification and Associated Ligands

Binding Site or Receptor	Subtype	Agonists	Antagonists
CHOLINERGIC			
	Nicotinic	*Acetylcholine[174] *Nicotine[175] Lobeline	*DBE[176] Pempidine Mecamylamine
	Muscarinic-1	McN-343-A *CMD[177] Oxotremorine	*Pirenzepine[178] *QNB[179] *N-Methylscopolamine[180]
	Muscarinic-2	*CMD[177] Oxotremorine	AFDX 116[222-226] *QNB[179] *N-Methylscopolamine[180]
HISTAMINE			
	H-1	2-Methylhistamine 2-Pyridylethylamine 2-Thiazolyethylamine	*Pyrilamine[143] Doxepin Iodobolpyramine Triprolidine Clemestine AHR 11325
	H-2	2-Methylhistamine Sopromidine Impromadine Dimaprit Betazole	*Cimetidine[144] Ranitidine BMY 25271 Impromidine Famotidine
	H-3	N^{α}-Methylhistamine αN^{α}-Dimethylhistamine $N^{\alpha}{}_{M}$-Chloromethyl-histamine	Impromidine[214]
ADENOSINE			
	A-1	*2-Chloroadenosine[154] *Cyclopentyladenosine[155] *Cyclohexyladenosine[156] *Phenylisopropyl-adenosine[157]	Theophylline PACPX *DPX[156] *XAC[158] DJB-KK[73]
	A-2	*NECA[159] CV 1808 MECA	Theophylline *DPX[156]
PEPTIDE			
	Opiate μ	Meptazinol[145] Oxymorphonazine *Dihydromorphine[146] *DAGO[147] Sufentanyl FK 33824	*β-FNA[219] Naloxonazine *Naloxone[146]
	δ	*DADLE[149] D-pen^{2}-D-pen^{5}-enkephalin DSLET	ICI 154,129 ICI 174,884

Table 2: Receptor Classification and Associated Ligands

Binding Site or Receptor	Subtype	Agonists	Antagonists
	κ	*U 69,593[150] U 50,488H[150] Dynorphin (1-17) *Tifluadom *Ethylketocyclazocine[151]	MR-2266 WIN-44,441-3
	σ	*SKF 10,047 *3-PPP Phencyclidine	
	ε	β-Endorphin	
Vasopressin		*Vasopressin[181]	
Substance P			
	SP-P	*Physalemin[182]	(D-Pro^2,D-Phe^7,D-Tryp^9)-SP
	SP-E	*Eledosin[183] Kassidin	
Cholecystokinin(CCK)		*CCK-4[184] *CCK-8 *CCK-33[185] Asperlicin	Proglumide[214] CR 1409[215] dbcGMP[216]
Somatostatin		*Somatostatin[187]	CycloAhep-Phe-Tyr-Lys-Thr[223] (B21)
Bombesin		*Bombesin[188]	
Neurotensin		*Neurotensin[217]	
TRH		MK 771 *TRH[189] *MeTRH[190] DN 1417	
Neuropeptide Y (NPY)		*NPY[191]	
Angiotensin II		*Angiotensin II[218]	
AMINO ACID			
γ-Aminobutyric Acid			
	A	*Muscimol[170] *GABA[171] *THIP[196]	*Bicuculline[172]
	B	*Baclofen[173]	
Chloride Channel			*Dihydropicrotoxinin[207] *TBPS[208]

Table 2: Receptor Classification and Associated Ligands

Binding Site or Receptor	Subtype	Agonists	Antagonists
	Glycine		*Strychnine[186]
	Glutamic Acid		
	EA-1	*Glutamate[167] NMDA Ibotinate ADCP	AP-7 *AP-5[167] AspAMP CPP[230]
	EA-2	*Kainate[168] Domoate *Glutamate[167]	Glu-tau GAMS
	EA-3	*AMPA[169] Quisqualate *Glutamate[167]	Glu-tau GDEE
LEUKOTRIENE			
		LTB_4[209] LTC_4[210] LTD_4[210]	FPL 55712[231]
DRUG			
	Benzodiazepine		
	'1'	*CL 218872[192] *Diazepam[193] *Zolpidem[194] *Clonazepam[197]	*Ro 15-1788[195] *CGS 8216[31]
	'2'	*Diazepam[193] *Clonazepam[197] CGS 9896	CGS 8216
	Peripheral	*Ro 5-4864[198]	*PK 11195[199]
	Phencyclidine	*PCP[200] *TCP[201] Tiletamine Dexoxadrol	Metaphit[225]
	Calcium Entry Blockers	*Nitrendipine[202] *Verapamil[203] *Diltiazem[204]	Bay K-8644
	+ Antidepressants	*Imipramine[205] *Desipramine[205]	
	+ Antitussive	*Dextromethorphan[206]	

Listed above is a select group of ligands shown to be active at the designated receptor site. This compilation is not intended to be comprehensive, but only representative. Inclusion does not imply specificity or even a high degree of selectivity for the receptor.

* Available as a radioligand

\+ The agonist-antagonist designation does not yet apply for these agents.

calcium as a second messenger had been underestimated in recent years because of difficulties associated with measuring this cation and because of the discovery of cyclic AMP.[62] The importance of calcium as a second messenger was, however, elegantly reviewed.[63] The discovery of the calcium entry blockers[64] and the phosphatidylinostiol /diacylglycerol/protein kinase C second messenger system[65] have rekindled interest in this ion.

(ii) Cyclic Nucleotides - The concept of second messenger systems was proposed in 1965 on the basis of findings associated with cyclic AMP,[62] a nucleotide produced from ATP by the action of adenylate cyclase. Cyclic AMP is thought to produce its effects by activation of kinases that catalyze the phosphorylation of intracellular proteins.[66,67] Another cyclic nucleotide thought to function as a second messenger is cyclic GMP,[68] which is formed from GTP by guanylate cyclase. Less is known about the manner in which cyclic GMP influences cellular activity.

Activation of certain neurotransmitter or hormone receptors promotes the production of cyclic AMP by stimulating adenylate cyclase which is coupled to the receptor recognition site by means of a regulatory protein.[69,70] Some receptor systems are negatively coupled to cyclase such that their activation reduces cyclic AMP formation. Alpha-adrenergic and adenosine receptor agonists may decrease or increase cyclase activity, depending upon the receptor subtype activated,[71-73] while opiates consistently decrease cyclic AMP production.[74] The intramembrane transducing element governing the interaction between the receptor and effector is the nucleotide regulatory protein (N or G), with Ns being a stimulatory protein and Ni being inhibitory.[70] The actions of the N protein are GTP-dependent and can be prevented by toxins; the Ni by pertussis toxin and Ns by cholera toxin. The N proteins are comprised of three subunits, alpha, beta and gamma. Ni and Ns differ only with respect to the composition of the alpha subunit, the site at which they are ribosylated by the toxins.[70]

Protein kinases are classified into three major subtypes; cyclic AMP-dependent, cyclic GMP-dependent and calcium-calmodulin-dependent.[66] There are various isoenzymes of these which determine their activity and specificity. Alterations in the phosphorylation state of various membrane-bound and soluble proteins, such as the beta-adrenoceptor,[55] have profound effects on their physiochemical properties, modifying their interactions with other cellular constituents, thereby altering function (i.e. receptor desensitization). Among the substrates for these kinases are synapsin I,[66] the nicotinic cholinergic receptor,[75] and tyrosine hydroxylase.[76]

(iii) Diacylglycerol (DAG) and inositol triphosphate (IP_3) - The dynamic nature of membrane-bound phospholipids has been known for years, leading to speculation they could function as receptor-coupled second messenger systems. Studies aimed at exploring this issue were complicated by the rapid changes

which occur in the composition of these membrane constituents. The discovery that lithium retards the metabolism of inositol phosphate (IP) has provided an important tool for research in this area.[77] The formation of IP_3 and DAG from phosphatidyl inositol is promoted by the activation of a number of receptors,[78] including alpha$_1$-adrenergic, dopamine, excitatory amino acid, histamine H-1, muscarinic M-1, serotonin-2, vasopressin-1, bradykinin and CCK sites. While the neuromodulator adenosine[73] has no effect on phospholipid turnover itself,[79] it augments the response to histamine.[80] As with the cyclic nucleotides, the phospholipid second messenger system is coupled to the receptor through an N protein, N_p.[78] IP_3 mobilizes calcium, while DAG stimulates protein kinase C,[232] with both actions resulting in an alteration in protein phosphorylation.[67] The tumor promoting phorbol esters mimick the effects of DAG in activating protein kinase C,[87] and have been used to label this enzyme.[82]

In addition to cyclic nucleotides, phospholipids and calcium, all of which affect protein phosphorylation, other second messenger systems have been proposed. These include the products of the eicosanoid pathways such as the prostaglandins and leukotrienes. The production of these substances may be associated with P-2 purinoceptor activation.[83]

Effector systems represent another target for drug action, a notion reinforced by the discovery that lithium, the most effective therapy for the treatment of bipolar affective illness, may act by altering the metabolism of receptor-mediated IP production.[77] While drugs acting to modulate second messenger production may be less selective than those acting at receptor recognition sites, it is possible such agents may be of use in correcting biochemical abnormalities at doses having little or no effect on normal tissue function.

Receptor Complexes - The discovery that specific receptors exist for some drugs led to the identification of a receptor for the anxiolytic diazepam.[35,85] Subsequent studies revealed that the high affinity benzodiazepine (BZ) recognition site is part of a complex comprised of a chloride ion channel and a GABA-A receptor recognition site. Receptor complexes have also been proposed for the calcium entry blockers[86] and for the excitatory amino acid N-methyl-D-aspartate.[87,88] The identification of the BZ receptor led to the discovery of inverse agonists and substances such as etazolate that potentiate receptor binding. Likewise, diltiazam enhances the binding of the dihydropyridine calcium entry blockers,[89] while the binding of phencyclidine, a dissociative anesthetic, is modulated by glutamate.[90] Given such progress it seems likely that other receptor complexes will be identified in the future. The potential to regulate receptor sensitivity and function indirectly is an exciting prospect when considering new approaches to drug development.

Endogenous Ligands - The discovery of the enkephalins and endorphins[8] has demonstrated that drugs do not necessarily produce their effects by directly modifying known neurotransmitter or effector systems. Moreover, while many endogenous modulators appear to be peptides, it has been shown that agents capable of interacting with their receptors need not be peptidergic. For example, diazepam and morphine interact with receptors for which the natural ligands are peptides.[35,84,85] Also, a non-peptidic cholecystokinin receptor antagonist has recently been isolated from bacterial fermentation broths.[91]

Given the technical simplicity of screening for endogenous substances using ligand binding assays, a potential exists for uncovering false leads. Some useful guidelines for assessing the validity of a claim that a substance is an endogenous ligand are provided (Table 3).

Table 3: Criteria for Evaluating the Pharmacological Significance of Novel Endogenous Receptor Ligands and Modulators

1. The extract or compound must be shown to have selectivity by its evaluation in at least three radioligand binding assays and must be examined for its proposed primary activity in an appropriate biological assay to rule out non-specific effects.

2. The activity of the extract or compound must be dose- or concentration-dependent. Unless there are substantiated bioavailability data, narrow 'windows' of biological activity should render the material suspect.

3. The isolation procedure and biological results must be confirmed in a separate laboratory.

4. A synthetic form of the isolated entity must have activity comparable to that of the natural substance and a series shown to have activity consistent with a structure-activity profile.

New Approaches To Receptor Modulators - The identification and characterization of receptors aids in the search for therapeutic entities which may, because of a mode of action different from that of existing compounds, offer new avenues for the treatment of disease. Many current drugs are pharmacologically active at a variety of receptors, some of which may not be associated with the illness being treated. For example, it is believed that the clinical response to neuroleptics is due to their dopamine receptor antagonist action.[92] However, this drug class also possesses anticholinergic and alpha-adrenoceptor blocking properties which contribute to their side effect profile (i.e. sedation, orthostatic hypotension, extrapyramidal symptomology).

Newer antipsycotics such as sulpiride and clozapine may be safer agents in that they are less likely to provoke extrapyramidal symptoms, either because they are selective for a subgroup of dopamine receptors which are most closely associated with the illness, or because they possess an action that counteracts the extrapyramidal side effects.

A novel approach to the treatment of schizophrenia involves the stimulation of dopamine receptors located on dopaminergic nerve terminals (autoreceptors).[35] When activated, these receptors reduce the release of dopamine, diminishing the dopaminergic tone which is thought to be overabundant in schizophrenia. A compound developed as an autoreceptor agonist was 3-PPP (N-N-propyl-3(3-hydroxyphenyl) piperidine).[94] In theory, this compound may lack those side effects associated with postsynaptic dopamine receptor blockade. However, 3-PPP was found to be a mixed agonist/antagonist,[95] and its presynaptic agonist action was attenuated within a week.[96] While 3-PPP proved not to be ideal, this approach is still being tested experimentally.[96]

As more selective agonists and antagonists are developed it will be possible to critically evaluate whether greater receptor selectivity improves therapeutic efficacy and reduces side effects. However, it is conceivable that a multiplicity of effects may be necessary for optimizing efficacy. Moreover, it is possibile that a drug appearing to be totally selective in standard laboratory tests may be less so *in vivo*, or may have an unusual action unsuspected from *in vitro* testing. For example, the histamine-receptor antagonist cimetidine was developed as a selective ligand for H_2 receptors in the fundus of the stomach.[98] However, it was recently shown that cimetidine enhances the binding of diazepam to central benzodiazepine receptors, raising the possiblity that a centrally-active analog may have anxiolytic potential.[99]

In general, receptor agonists have not been useful therapeutic agents. This may be due in part to the fact that the response to agonists tends to diminish over time, a phenomenon that is not completely understood. Because of this, emphasis has been placed on developing compounds that facilitate the actions of endogenous ligands. Monoamine oxidase and uptake inhibitors are examples of such agents.[100] By decreasing the metabolism or the removal of the neurotransmitter from the synaptic cleft, such drugs prolong the responses to these substances. In this context considerable effort is being expended on developing selective peptidase inhibitors to potentiate the effects of endogenous peptides.[101]

Neuromodulation - The distinction between neurotransmitters and neuromodulators is that the former have a short duration of action and, therefore, induce only transient changes in cellular communication. Examples are the monoamines, some amino acids and acetylcholine. Neuromodulators have a more prolonged effect on cellular activity and may therefore play an important role in such events as memory consolidation and

synaptogenesis. A list of neuromodulator candidates includes adenosine and the majority of neuroactive peptides.

Neurotransmitters and neuromodulators can co-exist in a single neuron, possibly invalidating the dogma associated with Dale's Law.[102] Examples are adenosine with acetylcholine,[103] cholecystokinin with dopamine,[104] alpha-melanocyte stimulating hormone with beta-endorphin[105] and neuropeptide Y with catecholamines.[106] Such co-transmission is now believed to be the rule rather than the exception. Four types of co-transmitter neurons have been postulated:[107]

1) those containing multiple neurotransmitters derived from a common gene coding for a prohormone,

2) those containing multiple neuropeptides derived from different genes,

3) those containing peptide and non-peptide neuro-effector agents, and

4) those containing multiple non-peptide neuro-transmitters.

The factors determining which co-transmitters will be present in a neuron are not known, although it is possible that the expression of one agent can cause the subsequent synthesis of a second. An example of co-effector modulation would be the heterologous desensitization phenomenon described previously. The functional significance of co-transmission has been considered,[107] with four modes of postsynaptic interaction proposed:

1) both co-effector agents influence the same receptor on the postsynaptic neuron,

2) each co-effector influences a different receptor on the postsynaptic neuron,

3) each co-effector influences a different receptor each of which are on a different neuron, and

4) co-effectors modulate the action of one another at the postsynaptic site.

Molecular Biological Approaches to Receptor Modulation - The most obvious contribution of molecular biology to pharmacology, in addition to the cloning and sequencing of receptors and immunomodulatory drugs,[3] has been in the utilization of monoclonal antibodies to produce large quantities of hormones such as insulin. Molecular biology also holds great promise as a means for understanding the factors related to immune competence[108] and its relationship to disease.[109] The use of antiidiotypic antibodies as selective receptor antagonists, while capturing the imagination,[111,112] should be balanced

against the feasibility of transporting such entities to their target areas without degradation or the triggering of an immune response. While the elegant work of Herbert and others[113] has indicated that it may be possible to manipulate transcriptional and translational processes with drugs, much needs to be learned about the design of therapeutic agents that will selectively interact with the system of interest.

Drug Receptors - Receptor-based drug discovery has been concerned mainly with the cholinergic and monaminergic transmission processes.[114] With the discovery of the enkephalins and the identification of the central benzodiazepine receptor, attention has been directed toward the possibility that many therapeutic agents may have their own recognition site independent of any known neurotransmitter or neuromodulator receptor. This theory assumes the existence of endogenous entities for which these therapeutic agents substitute or inhibit. As an underlying genetic code the ability to synthesize an antagonist of the peripheral cholcystokinin receptor and, also, why plants would make so many agents (i.e. opiates, cardiac glycosides) of critical use in the treatment of human illness.[115] Recognition sites for tricyclic antidepressants, psychotomimetics such as phencyclidine,[116] anticonvulsants, barbiturates[117] and amphetamines[118] have been reported. Whether or not they represent receptors for hitherto unidentified endogenous substances remains unknown.

Summary - Receptors are critical for mediating the actions of hormones, neurotransmitters and other agents that modify cellular activity. Receptors are also a major focus for drug design and development since they are responsible for imparting selectivity to the actions of these substances. Technical advances during the past decade have made it possible to more fully characterize receptor sites in terms of their chemical composition and kinetic properties, as well as with respect to the factors that regulate their number and function. Such studies have led to the discovery of new endogenous agents and to the realization that drugs need not attach directly to receptor recognition sites to influence their responsiveness. These findings are providing a new perspective with regard to drug mechanisms fostering the development of novel drugs. Given the practical implications of these advances in biology, it is important that medical chemists have some familiarity with this topic as they design the next generation of therapeutic agents.

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Chapter 22. Mitogenic Factors as Oncogene Products

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Introduction- The proliferation and metabolic activity of normal mammalian cells in culture is regulated by polypeptide growth factors and other hormones whose action is mediated by specific cellular receptors. The availability of purified growth factors has enabled investigators to study their effects on cell growth in culture, and to begin to elucidate the sequence of events leading to DNA synthesis and cell proliferation. These studies established a new class of hormonal polypeptides, known collectively as growth factors, as the regulators of normal cell growth.[1]

While the function of growth factors has been associated with the regulation of normal cell growth, uncontrolled cell proliferation, characteristic of the transformed state, became the domain of oncogenes. Acute transforming retroviruses contain discrete genomic sequences acquired by recombination between the genome of the virus and host cellular gene sequences. When incorporated within the retrovirus genome such transduced cellular sequences, termed viral oncogenes (v-onc), acquire the ability to induce neoplastic transformation. About twenty v-onc genes have been identified (reviewed in refs 2,3). Some of the same sequences homologous to the transforming retroviral genes have been recognized in normal untransformed cells. These cellular oncogenes or proto-oncogenes, are targets of structural and regulatory alterations that may lead normal cells to become malignant. Point mutation, chromosomal translocation and oncogene amplification have been implicated in the transforming potential of cellular genes[4-10]. Despite this understanding, little is known about the functional role of proto-oncogenes and of their altered counterparts in inducing uncontrolled cell proliferation.

Recently, studies on growth factors and their cell membrane receptors have provided a direct link between oncogenes and a known biologic function. A striking example is provided by the recognition that the oncogene, v-sis, of the simian sarcoma virus (SSV), an acute transforming retrovirus of primate origin, encodes for a potent mitogen, the platelet-derived growth factor (PDGF). This suggests that the transforming properties of SSV derive from the constitutive expression of this potent mitogen, PDGF. In another development, the avian erythroblastosis virus (v-erb-B) oncogene has been shown to encode a truncated form of the epidermal growth factor (EGF) receptor. More recently, evidence has been presented for a possible relationship between the c-fms proto-oncogene product and the receptor for the mononuclear phagocyte growth factor, CSF-1. These findings, linking oncogenes to functional products which are involved in cell regulation, provided a new understanding of the potential role of oncogenes in neoplastic transformation.

Nature and Function of PDGF - PDGF is the major growth factor of human serum. It is a potent mitogen for the growth of mesenchymal derived cells in culture, such as diploid fibroblasts, arterial smooth muscle cells, and brain glial cells. It is stored in the α-granules of platelets, and it is released from platelets into the serum during blood clotting. In vivo, PDGF is apparently synthesized by megakaryocytes and stored and transported by platelets. These cells have an affinity for injured sites aggregating there and releasing their contents. In this manner, the platelets selectively deliver PDGF to sites of injury where it stimulates wound repair (reviewed in refs 11-15).

PDGF has been purified initially from clotted human blood serum,[16] and subsequently from clinically outdated human platelets,[17,18] or platelet-rich plasma.[19,20] The molecular weight of biologically active, unreduced PDGF was estimated at 28-35 kD. Reduction resulted in the appearance of lower molecular weight, inactive polypeptides ranging from 12 kD to 18 kD, suggesting that active PDGF consists of two polypeptide chains.[13-15] The aminoterminal amino acid sequence analysis of PDGF provided evidence that it consists of two nonidentical but structurally related polypeptide chains (PDGF-1 and PDGF-2) linked together by disulfide bonds.[21,22].

The primary function of PDGF is the stimulation of DNA replication and cell division of diploid fibroblasts, arterial smooth muscle cells, and brain glial cells. In addition, PDGF has been shown to be a potent chemoattractant for cultured arterial smooth muscle cells, fibroblasts, neutrophils, and monocytes. Other functions of PDGF include the ability to stimulate important metabolic processes, including protein, lipid, and prostaglandin synthesis and stimulation of tyrosine kinase activity (reviewed in refs. 11-15).

Regulation of Cell Growth by PDGF. The isolation of PDGF enabled extensive studies on its mode of action in cell culture. These studies revealed that the transition between the G_0/G_1, phase of the cell cycle and the S-phase required the synergistic effects of both PDGF and of other hormones present in platelet-poor plasma.[23-25] Some of these hormones in plasma were shown to belong to the family of polypeptides with insulin-like activity.[25] These studies provided a new understanding of cell growth control. The significant finding was that the transition from the Go/G1 phase to the S-phase of the cell cycle could be subdivided into two stages. One, called competency, is controlled by PDGF and allows cells to enter the Go/G1 phase of the cell cycle.[23,24] The other called progression, is controlled by factors in plasma that enable the progression of the PDGF-induced competent cells into the S-phase.[25] The PDGF-induced competency could be achieved by a brief exposure of the cultured cells to PDGF. For example, cells exposed to PDGF remained competent for up to 13 hours after PDGF was removed from the cell culture. Addition of plasma to the cultures pre-exposed to PDGF enabled the progression into S-phase. These studies imply that PDGF is the limiting factor for the in vivo growth of normal target cells. Progression factors are available in vivo from circulating blood. In contrast, PDGF is transported by platelets and it is available only selectively, in small amounts, at the site of injury during platelet degranulation. For this reason, target cells to PDGF action that acquire the ability to synthesize their own supply of PDGF escape this regulatory restriction, and they are capable of entering into a continuous and unregulated growth.[15]

As described above, a transient exposure of cultured cells to PDGF could render the cells competent to enter the cell cycle. This implies that PDGF induces stable, secondary "modulators" in its target cells that render them capable of responding to progression factors. Such modulators could be specific, PDGF-induced RNAs and/or proteins.[26-28] Recent studies provided evidence that PDGF can induce c-myc mRNA[29] and c-fos mRNA in cultures of 3T3 fibroblasts, suggesting that gene products of both oncogenes may be involved in the progression of cells through the cell cycle.[30-34]

The SSV Oncogene, v-sis, Codes for the PDGF-2 Polypeptide Chain - Elucidation of the aminoterminal amino acid sequence of human PDGF[21] revealed a near identity between the amino acid sequence of the PDGF-2 polypeptide chain and the transforming gene product of the simian sarcoma virus.[35-36] This recognition provided the important information that the SSV oncogene, v-sis, encodes the PDGF-2 polypeptide chain. This finding suggested that the ability of the simian sarcoma virus to induce transformation derives from the incorporation of the PDGF-2 gene within the retroviral genome. The resulting transforming oncogene region within the retrovirus genome codes for a PDGF-2 mitogen and is capable of inducing unregulated growth by the continuous production of this potent mitogen.

The simian sarcoma virus is an acute transforming retrovirus which was initially isolated from a fibrosarcoma of a wooly monkey, and it is the only sarcoma virus of primate origin.[37] Characterization of its genome localized its transforming gene to a cell-derived onc sequence, v-sis.[38,39] The v-sis gene has been sequenced[40] and its 28 kD transforming protein product ($p28^{sis}$) has been identified by immunoprecipitation with antisera prepared against synthetic polypeptides corresponding to its aminoterminal and carboxyterminal regions.[41]

Structural and Functional Identity Between the sis/PDGF-2 Oncogene Product and PDGF - The transforming protein product, $p28^{sis}$, of the SSV oncogene v-sis, consists of a single chain polypeptide containing 226 amino acid residues. The region corresponding to the PDGF-2 chain starts at the serine residue in position 67, which follows a double basic sequence (lys-arg) at position 65-66. This is apparently the processing point yielding a polypeptide of 160 residues with a molecular weight of 18,056, essentially the same size estimated for the PDGF-2 chain on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[21] However, the SSV oncogene codes only for one chain (PDGF-2) of the PDGF polypeptide.[35] Previous studies have shown that disruption of the disulfide-linked dimeric structure of PDGF resulted in a total loss of activity, suggesting that the single reduced chains are biologically inactive.[17] For this reason, it was important to establish whether the v-sis oncogene product was functioning as a single PDGF-2 polypeptide chain or as a disulfide-linked dimer similar to biologically active PDGF. This important question was resolved by the demonstration that in SSV-transformed cells, $p28^{sis}$ undergoes a series of discrete processing steps, including disulfide-linked dimer formation and proteolytic cleavage, to yield PDGF-2 homodimers structurally and immunologically resembling the disulfide-linked dimeric form of biologically active PDGF.[42] The precursor polypeptide, $p28^{sis}$, is synthesized by cell membrane bound polyribosomes, followed by dimerization in the endoplasmic reticulum and processing at the cell periphery to yield structures analogous to biologically active PDGF.

The functions of the PDGF homodimer are similar to those of biologically active PDGF. Secretory products of PDGF-2 homodimer obtained from the conditioned media of SSV-transformed NRK cells were capable of stimulating [^{3}H]thymidine incorporation in cultured 3T3 cells, cell-membrane protein phosphorylation, at tyrosine residue, in human fibroblasts, and binding to specific cell-membrane PDGF receptors.[44] Like PDGF, the biologic activity of the PDGF-2 homodimer was shown to be heat-stable and sensitive to reducing agents.[44]

The mechanism of action of the PDGF-2 homodimer synthesized by SSV-transformed cells is as yet unclear. A biologically active 34 kD PDGF-2 homodimer is secreted by SSV-transformed NRK cells.[44] PDGF-receptors have been demonstrated on these cells,[44] suggesting that self-stimulation of the SSV-transformed NRK cells may be mediated by an autocrine mechanism involving the release of PDGF-2 homodimer and stimulation of growth through binding to PDGF cell-membrane receptors. Secretion of the v-sis/PDGF-2 oncogene products by SSV-transformed cells is consistent with the presence of a signal sequence in the p28^{sis} protein, derived from the envelope gene of the parental retrovirus. Deletion of the signal sequence of the env-sis coding region has been correlated with loss of biologic activity, suggesting that the v-sis product must enter the cellular secretory pathway for expression of biologic activity.[45] However, in SSV-transformed marmoset cells (HF/SSV), the v-sis/PDGF-2 oncogene products were localized in the cell membrane fraction, without evidence of secretory products.[46] This suggested the possibility that, at least in these cells, the mitogenic proteins act through a direct interaction with membrane PDGF receptors without prior secretion. Additional evidence is required for the elucidation of the mode of action of the v-sis/PDGF-2 oncogene products.

c-sis gene, which is the normal homolog of the v-sis, was also shown to encode the PDGF-2 chain.[47,48] The presence of sis-related messenger RNAs in human tumors of mesenchymal origin has been reported.[49-51] The human osteosarcoma cell line (U2OS) was shown to synthesize two c-sis mRNA species of 4.0 Kb and 3.7 Kb in length, with a minor species of about 2.6 Kb.[50] The concentration of each c-sis transcript was estimated at about 10-20 copies per U2-OS cell. The human glioblastoma (A172) and fibrosarcoma (HT1080) cell lines were shown to synthesize a 4.4 Kb mRNA that contained sequences from all the six identified exons of the human c-sis gene.[51] In some human T-cell leukemic virus (HTLV)-infected cell lines, sis-related mRNAs were also detected.[52] The presence of the activated c-sis gene in these human malignant cell lines was accompanied by the synthesis and secretion of biologically active PDGF-like polypeptides. The properties of these c-sis/PDGF-2 gene products appear to be similar to those of biologically active PDGF, and the v-sis oncogene products synthesized by SSV-transformed cells.[50,51,53,54]

Activation of the c-sis gene and synthesis and secretion of PDGF-like mitogen by human malignant cells of mesenchymal origin suggests the possibility that sis activation in these cells might be involved in the processes leading normal cells of mesenchymal origin towards malignancy. The normal counterparts of these malgnant cells are target cells to PDGF action. In vivo, the growth of these cells depends on the delivery of PDGF during platelet degranulation. The ability of the malignant cells to produce their own, continuous supply of PDGF, bypasses this controlling mechanism, and may lead to uncontrolled cell proliferation.

c-sis Gene Expression in Nontransformed Cells - As described above, sis activation and production of PDGF-like mitogen has been associated with the transformed state. Recent studies demonstrated that certain un-transformed cell lines exhibit c-sis transcripts and have the ability to synthesize and to secrete active PDGF-like polypeptides. Some of these cell lines are not targets to PDGF action. For example, the presence of c-sis transcripts and production of PDGF-like mitogen have been demonstrated in cultured human or bovine endothelial cells. Sequence analysis of a sis homologous complementary DNA clone from human endothelial cells demonstrated that these cells express the PDGF-2 polypeptide chain of human PDGF.[58] Activated monocytes and macrophages also exhibit c-sis transcripts and are capable of producing PDGF-like mitogen.[59,60] Recent studies have shown that phorbol ester-induced differentiation of myeloid leukemic cells along the monocyte-macrophage lineage, is associated with the induction of the expression of c-sis gene and the synthesis and secretion of PDGF-like mitogen.[61] The ability of endothelial cells, monocytes, and macrophages to secrete PDGF-like mitogen may serve important paracrine functions in vessel neogenesis, inflammation and wound healing.

Expression of the c-sis gene and secretion of PDGF-like mitogen has been shown in human placenta trophoblasts,[62] and in fetal and neonatal smooth muscle cells.[63] These cells grow rapidly in a fashion resembling transformed cells. It is possible that the production of PDGF-like polypeptides by these cells contributes, respectively, to the growth of the developing placenta,[62] or to the development and maturation of aorta.[63]

In summary, the discovery that the sis oncogene encodes for a potent mitogen established a functional role for this oncogene, and provided a basis for the understanding of its role in neoplastic transformation. The presence of this proto-oncogene in certain un-transformed cells may serve important physiological processes mediated by the paracrine action of the c-sis/PDGF-2 gene products.

The EGF Receptor and the v-erb-B Oncogene of Avian Erythroblastosis Virus (AEV) - Epidermal growth factor (EGF) is one of the earliest discovered and characterized polypeptide growth factors.[64] It was initially isolated from extracts of mouse submaxillary glands and was shown to accelerate eyelid opening in newborn mice.[64] Subsequent studies demonstrated that EGF is a potent mitogen for a variety of cell lines.[65] The biologic effects of EGF are expressed by binding to specific membrane receptors of target cells. The receptor contains an intrinsic tyrosine kinase activity that is induced following EGF binding.[65-67] The EGF receptor complexes are rapidly internalized by the cell[68] and the internalized EGF is apparently degraded in the lysosomes.[68]

Stimulation of tyrosine-specific kinase activity by EGF has been suggested to represent an important early step in triggering DNA synthesis and cell proliferation. The recognition that retroviral transforming proteins[69] and other growth factors such as PDGF,[13,14] insulin,[70,71] and insulin-like growth factor (IGF-I)[72] promote cell growth and share in their specificity of stimulating tyrosine-specific kinases has generated a strong interest for a possible connection between cellular protein tyrosine phosphorylation and cellular growth. However, there is no conclusive evidence as yet, linking protein tyrosine phosphorylation to cell proliferation.

EGF Receptor - The EGF receptor is a glycosylated transmembrane protein with a molecular weight of 170-175 kD. Amino acid sequence analysis of peptide fragments derived from purified human EGF receptor revealed a significant homology with the deduced sequence of the v-erb-B transforming protein of AEV.[73] Subsequently, the entire cDNA and predicted amino acid sequence has been reported, demonstrating that the human EGF receptor precursor consists of 1,210 amino acids.[74] Cleavage of a signal peptide consisting of 24 amino acids yields a receptor protein of 1,186 amino acids. The receptor has a glycosylated extracellular EFG-binding domain and a cytoplasmic domain containing the tyrosine protein kinase activity of the receptor. The two domains are separated by a single transmembrane region of 23 amino acids.[74-76]

The extracellular EGF-binding domain consists of 621 amino acids and it contains the amino-terminal region while the carboxyterminal region of 542 amino acids is the cytoplasmic domain. Extensive amino acid sequence homology has been shown between the carboxyterminal cytoplasmic region of the EGF receptor and the transforming protein of the v-erb-B oncogene. This homology includes a region of 376 amino acid residues beginning at the cytoplasmic junction of the receptor and including the transmembrane domain of 23 amino acid in both sequences. A 244 amino acid region of the cytoplasmic domain contains 60 amino acid residues which are conserved in other protein kinases including the members of the src gene family of tyrosine-phosphorylating kinases.[77] They include the tyrosine which is the substrate for the autophosphorylation reaction of pp60^{src}.

These studies demonstrated that the transforming protein of the v-erb-B oncogene shares extensive amino acid sequence homology with the cytoplasmic region of the EGF receptor which contains the tyrosine protein kinase domain. It seems that the v-erb-B protein is a truncated form of the EGF receptor, lacking the extracellular EGF-binding domain, but retaining the transmembrane region. The glycosylated form of the v-erb-B protein is membrane associated and has a molecular weight of 64-68 kD.[78,79] Further processing of the carbohydrate side chain occurs in the Golgi apparatus and part of the transforming protein is present in the cell surface with a molecular weight of 75-80 kD.

The findings described above raise several important questions. For example, the nature of the signal transduction between the two functional EGF receptor domains across the membrane is unknown. EGF-receptor binding of the extracellular domain transduces a signal that results in stimulation of tyrosine kinase activity and autophosphorylation. The possibility has been suggested[74] that EGF induces receptor clustering of the extracellular domain. This may bring the cytoplasmic domains into close proximity resulting in inter-receptor interactions which might include stimulation of tyrosine kinase activity and autophosphorylation. The functional role of the v-erb-B protein is still unresolved. It is possible that this truncated form of the EGF receptor mimics an occupied EGF receptor in inducing transformation. However, there is no conclusive evidence that the v-erb-B protein has protein kinase activity in vitro. The lack of EGF receptors in erythroblasts makes difficult a direct connection between the growth of these cells and the concept of the v-erb-B protein as an activated EGF receptor.

Relationship Between the Receptor for the Mononuclear Phagocyte Growth Factor (CSF-1) and the c-fms Proto-Oncogene Product - The viral oncogene

(v-fms) of the McDonough strain of feline sarcoma virus encodes a transmembrane glycoprotein of 140 kD.[80-83] This glycoprotein has the properties of a cell surface receptor, and its expression on the cell surface is required for transformation.[84] Like the EGF receptor, the v-fms product has a glycosylated extracellular aminoterminal domain and a cytoplasmic carboxyterminal domain which is associated with tyrosine protein kinase activity.[83,85,86] The cellular (c-fms) proto-oncogene product is a 170 kD glycoprotein[87] which crossreacts with antisera to a recombinant v-fms-coded polypeptide.[88] The c-fms glycoprotein product is expressed at high levels in mature macrophages. These differentiated cells also contain a 165 kD glycosylated receptor protein which binds the murine colony stimulating factor (CSF-1). Binding of the CSF-1 to its receptor stimulates tyrosine-specific protein kinase activity. CSF-1 stimulates hematopoietic precursor cells to form colonies containing phagocytes.[89] It binds to mononuclear phagocytes and their precursors and to macrophage and myelomonocytic cell lines in culture. Antisera to v-fms-coded polypeptide, which crossreact with the c-fms gene product, also reacted with the murine CSF-1 receptor.[88] This suggests a close relationship between the c-fms proto-oncogene product and the CSF-1 receptor. Sequence information of the CSF-1 receptor will provide a conclusive confirmation of this relationship.

Summary - Recent reports linking oncogenes to functional products which are involved in cell growth regulation, provided a rational basis for the understanding of the role of oncogenes in transformation. A striking example is provided by the discovery that the sis oncogene encodes for one of the polypeptide chains of a potent mitogen, PDGF. Intracellular processing of the sis oncogene polypeptides results in the production of mitogenic PDGF-like homodimers. In another development, the receptor of EGF, a potent mitogen, was shown to have extensive homology with the v-erb-B oncogene product which seems to encode the tyrosine protein kinase domain of the EGF receptor. This truncated form of EGF receptor may act as an occupied EGF receptor in inducing transformation. Homology was also noticed between the tyrosine kinase region of the EGF receptor and other protein kinases which are members of src gene family. A connection between the receptor of the colony stimulating factor and the c-fms protein product has been suggested on the basis of immunocrossreactivity between the c-fms product and the CSF-1 receptor with antisera to a v-fms-coded polypeptide.

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Chapter 23. Nucleotide Metabolism in Parasitic Protozoa

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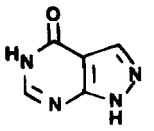
Introduction - A variety of human and animal diseases are caused by parasitic protozoa including malaria, Chagas disease (South American trypanosomiasis), African trypanosomiasis, giardiasis, leishmaniasis, coccidiosis and toxoplasmosis. The nucleotide metabolism of these organisms often differs strikingly from the metabolism of the host mammalian cells.[1] For example, it is commonly found that parasitic protozoa lack de novo biosynthesis of purines and are therefore metabolically dependent upon the host for the synthesis of purine bases.[1] At another level, even when the host and parasite have the same enzyme present, the specificity for a given molecule as a substrate or inhibitor can be vastly different, giving rise to opportunities for selective toxicity.[1] This review covers some of the major chemotherapeutic studies carried out on parasitic protozoa recently in the area of purine or pyrimidine nucleotide metabolism. The designation R after a compound number stands for β-D-riboside, RP for the 5' monophosphate and RP_3, for the 5' triphosphate.

Pyrazolopyrimidine Analogs - Derivatives of pyrazolopyrimidine, including the parent compound allopurinol, 1, have been intensively studied because they are metabolized in an unusual way by pathogenic parasitic protozoa of the genera Leishmania and Trypanosoma[2], which results in antiparasitic activity. Leishmania promastigotes form millimolar concentrations of the ribonucleotide 1RP, which is then converted into the aminated AMP analog 2RP, followed by conversion to $2RP_3$ and then incorporation into RNA.[2] Several factors contribute to the ability of 1 to be incorporated in this manner, in contrast to mammalian cells which neither convert 1RP to 2RP nor incorporate 1 into RNA. The parasite has high levels of hypoxanthine guanine phophoribosyl transferase, HGPRTase, which phosphoribosylates the purine analog, and also lacks xanthine oxidase to catalyze oxidation to oxypurinol, 3, which occurs readily in mammalian cells.



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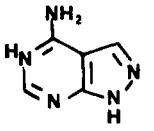

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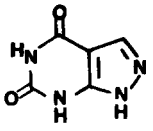


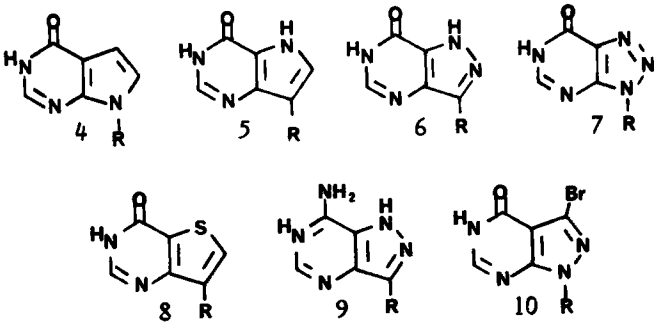
3

The succino-AMP synthetase and succino-AMP lyase, which normally carry out the conversion of IMP to AMP, have been shown to convert 1-RP to 2-RP if the enzyme from Leishmania donovani or Trypanosoma cruzi is used, whereas no conversion occurs with the mammalian enzyme.[3,4] The K_m values are higher

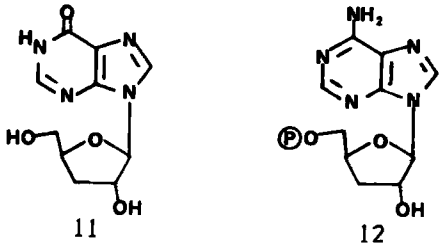
and V_{max} values less than 1% of the corresponding values for IMP with the succino-AMP synthetase from the parasites. However, the very high levels of 1RP compared to IMP help to overcome this deficit and allow substantial conversion to 2RP. Leishmania also very readily convert 2, the amino analog of 1, into 2RP and then 1RP due to the action of AMP deaminase. Therefore, the efficacy of 2 against these organisms, can still depend upon this selectivity of succino-AMP synthetase which creates a steady state concentration of 2RP for RNA incorporation.[2] The effects of 1 on Leishmania can be reversed by the addition of adenine, but this reversal does not occur if 1 is allowed to metabolize prior to the addition of adenine.[2]

A number of studies have tested the efficacy of either 1 or 2 against Leishmania and Trypanosoma. In culture, T. cruzi aminates 1RP to 2RP and is sensitive to 1 whereas non-pathogenic T. rangeli does not aminate 1RP and is insensitive to 1.[5] T. rangeli is slightly sensitive to 2 with low concentrations of adenine in the medium. Many newly isolated strains of T. cruzi were insensitive to 1 and 2 but became sensitive after in vitro passage. This change was due to a difference in drug uptake rates rather than measurable differences in HGPRT, APRT or succino-AMP synthetase activities.[6] Treatment of T. cruzi infections in mice with 2 at 0.25 mg/kg/day was effective even though this was 150-fold lower than the effective dose for 1.[7] Using radiolabelled 1, it was demonstrated that blood stream and intracellular forms of T. cruzi metabolize the drug as do the epimastigotes in vitro and that 1 was effective in controlling the infection in tissue culture.[8] Another in vitro study showed 2 to be six fold more active than 1 against T. cruzi.[9] This same study included a successful in vivo test of 2 (0.125-0.5 mg/kg daily for 10 days) against a virulent strain of T. cruzi in two different strains of mice. The drug decreased parasitemia and increased survival time. In another study, 1 had no apparent affect on the course of infection by T. cruzi in mice when administered at 0.3 or 0.75 mg/kg orally or i.p.,[10] although earlier studies did indicate activity.[11,12] When tested against promastigotes of 10 isolates of Leishmania, 2 was several-fold more effective than 1.[13] Only 50% of Leishmania tropica amastigotes in human macrophages were eliminated by achievable serum levels of 1 under conditions where therapeutically useful anti leishmanial agents are capable of clearing 90%.[14] Previously a study of Leishmania major and Leishmania mexicana amazonensis demonstrated oral efficacy of 1 in mice.[15] A clinical study on antimony resistant visceral leishmaniasis in humans using 1 gave apparent cures in 4 of 6 patients.[16] Several other reviews of pyrazolopyrimidine metabolism in leishmania and trypanosomes have appeared.[17,18]

Inosine Analogs - The riboside of allopurinol, 1R does not get readily metabolized by xanthine oxidase (as does 1) and is more effective than 1 against some species of leishmania.[19] It is 10-fold more active than 1 against L. braziliensis and 300-fold more active against L. donovani but equivalent when directed against L. mexicana. Conversion of 1R to 1RP occurs by the action of a nucleoside phosphotransferase rather than through a kinase.[19] Under conditions that converted inosine to hypoxanthine no conversion of 1R to 1 occurred. T. cruzi Costa Rica strain was little affected by 1R and failed to convert 1R to 1RP. However, this appears to be highly dependent upon the strain since Peru and Y strains are sensitive and do convert 1R to 1RP.[20] A variety of inosine analogs, 4-8, have been synthesized and tested against L. donovani, T. cruzi and T. gambiensi.[21] With the exception of 7, all showed considerable activity with relatively little toxicity for mouse L cells.



The effective doses were comparable to 1R (1-10 uM). Compound 6R was extraordinarily active against T. gambiense whereas 1R is relatively inactive against this organism. The 3-bromo analog of 1R, 10R was also more active than the parent compound against L. tropica within human macrophages in vitro.[22] In a study in which a variety of nucleoside analogs were tested against L. tropica, in vitro, 3'-deoxyinosine, 11, and several tubercidin derivatives were active against promastigotes.[23,24] Leishmania phosphorylate and then aminate 11 to give toxic 3'-deoxyadenosine nucleotides (cordycepin), 12. The EC_{50} value is 4×10^{-7} M



against promastigotes but it is much less toxic (EC_{50} 10^{-4} M) towards mouse mammary tumor FM3A cells. In L. donovani and in mouse L cells, 4R, 7R, and 6R (Formycin B) were all converted to their respective ATP analogs.[25] Compound 7R was converted into the GTP analog by L cells but not by L. donovani. The parasite metabolized 5R and 1R to the ATP analogs and also metabolized 5R to the GTP analog. Formycin B (6R), 1R and 5R were phosphorylated by two separate routes in mouse L cells as evidenced by differential inhibition of phosphorylation of these compounds by an adenosine kinase inhibitor. L. donovani, by contrast, metabolizes 6R in a manner unaffected by this same inhibitor. The ability of the organism to convert these compounds to the ATP or GTP analogs correlates well with cytotoxicity.[24] In a similar study, seven inosine analogs, including analogs of 9R and analogs of 4R in which substituents were placed at the 7 position and inosine analogs with a modified sugar were shown to have selective toxicity toward L. tropica compared to mouse L1210 cells.[26] The metabolism of 4R to adenosine nucleotides was carried out by L. tropica.

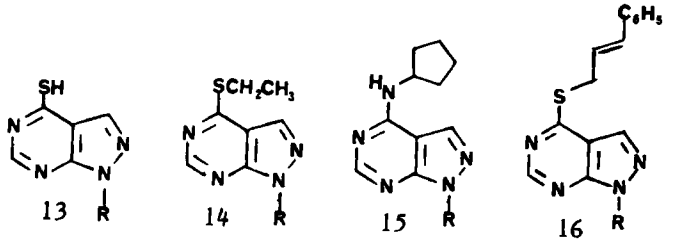
Formycin B - Considerable effort has been put into studying the mode of action of Formycin B, 6R, as an antiparasitic agent, since the compound is not phosphorylated or cleaved in mammalian cells.[2] At 1 uM, L. donovani and L. mexicana promastigotes are inhibited by 50%, as are L. mexicana amastigotes in macrophages. The compound was also effective against amastigotes in livers

of infected hamsters. Further studies confirmed the antiparasitic activity and also demonstrated the conversion by the parasite of 6R to 6RP and then to the AMP analog 9RP.[28] The parasite succino-AMP synthetase accepted 6RP as a substrate with a K_m of 26 uM and a V_{max} that was 1% of the value for IMP. Mammalian succino-AMP synthetase was capable of using 6RP as a substrate with a V_{max} 40% of that for IMP. The 9RP produced by this activation process was incorporated into cellular RNA. Both 6RP and 1RP were inhibitors of GMP reductase from leishmania. Treatment of promastigotes of L. donovani with either 1 or 6R resulted in a > 95% decrease in incorporation of ^{14}C-xanthine into GTP. Other studies have demonstrated the efficacy of 6R against L. mexicana, L. braziliensis and L. donovani, and confirmed the metabolism to toxic adenine nucleotide analogs.[29] A similar metabolic fate of 6R was also demonstrated with cutaneous Leishmania amastigotes within cultured human macrophages.[30] Uninfected macrophages also converted 6R into the same metabolites but to a much lower level. In vitro testing[31] of 6R, 6RP, 9R, 9RP and $6RP_2$ demonstrated activity against L. tropica in macrophages with ED_{50} values of .02-.04 uM whereas the corresponding value for 1R was 190 uM. However, antileishmanial activity generally paralleled toxicity toward the macrophage host. Over an 8 hour period, accumulation of metabolites from 6R were studied in T. cruzi and $9RP_2$ was formed with a progressive incorporation of labelled 6R into RNA.[32] No label was found in DNA, nor were nucleotide pool levels altered, so that T. cruzi metabolism of 6R is analogous to that found for leishmania.

When L. tropica promastigotes were subjected to progressive increases in 6R concentration (0.2-100 uM) highly resistant mutants were obtained which retained the resistance for numerous generations in the absence of the drug.[33] The resistance may be due to alteration of membrane proteins responsible for the transport of 6R. Another study of 6R resistant L. donovani demonstrated defective transport of 6R in the resistant mutants.[34] These mutants are also cross resistant to growth inhibition by 9R even though wild type and resistant populations incorporate ^{3}H- 9R equally well into 9RP and $9RP_2$ pools as well as into RNA. This suggests that the antileishmanial activity of 6R is not due to its metabolism to 9R. An alternative proposal for the mode of action of 6R is that 6R metabolites cause the depletion of an essential intracellular metabolite.[34] In contrast, however, in L. mexicana conversion of 6R into 9R metabolites and then into RNA was established, and Formycin A metabolites were demonstrated to inhibit protein synthesis.[35] Additionally, messenger RNA was translated only 40% as efficiently as control mRNA, leading to the conclusion that at least part of antileishmanial activity of 6R was due to conversion to 9R metabolites that inhibited protein synthesis. In another study, it was shown that human red blood cells took up 9R and metabolized it to $9RP_2$ and that Leishmania-infected macrophages phagocytized IgG-coated red blood cells containing the $9RP_2$.[36] This treatment suppressed the multiplication of the organisms by 80%.[3] The ED_{50} for suppression was .02 uM for the encapulated drug compared to 0.84 uM for the unencapsulated form, suggesting that this formulation may be efficacious in vivo.

Thiopurinol Analogs - Thiopurinol, 13, and its riboside have also shown some promise as antiparasitic agents.[37] Like 1, 13 is metabolized to 13RP but then no further, and the anti leishmanial activity of 13 is comparable to 1. The ribosides, 13R and 1R are also comparable to each other and more active than the bases. L. donovani converts 13 to 13RP but not to the di or triphosphate forms. No conversion to 1RP or 2RP occurred and the thiol group and the ribose remained bound to the 13 base. The 13 base is a substrate for HGPRTase,

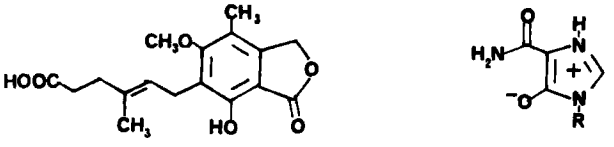
but the product 13RP was not a substrate for succino AMP synthetase.[38] The phosphorylation of 13 occurred by a nucleoside phosphotransferase as had been demonstrated for 1R. Even though 13RP was not a substrate for succino-AMP synthetase it did act as an inhibitor for the reaction of the enzyme with IMP. The sensitivity towards 13 or 13R by T. cruzi is highly strain dependent with some insensitive and others generating high concentrations of 13RP which inhibits the T. cruzi succino-AMP synthetase.[2] Adenine reverses the activity, as expected if the putative block of AMP synthesis is subverted by direct synthesis of AMP through APRTase using adenine.[39] Compound 13 has no activity against Eimeria tenella in vivo in chicks, whereas 13R did show some activity.[40] A series of alkyl-thiopyrazolopyrimidines



13 14 15 16

were prepared from 13R by alkylation and the ethylthio derivative 14R was active as an anticoccidial agent at 50 ppm. in feed against E. tenella. Deletion of the ribose, alteration of substituants in the 2-position or making 3-deaza or 8-aza modifications decreased activity. A series of 4-alkylamino derivatives were also prepared with 15R being the most active at 200 ppm.[41] A further series of alkyl-thio derivatives were prepared of which 16R was the most effective and showed the broadest spectrum of activity against other Eimeria species.[42] However, control of E. acervulina was not achieved with any of the derivatives. Purification of the adenosine kinase from E. tenella, E. brunetti and E. acervulina as well as chick liver was accomplished and comparative studies revealed considerable differences in inhibition and substrate specficity.[43] Pyrazolopyrimidine nucleosides fell into two groups with respect to inhibitory ability and that grouping correlated with anticoccidial activity. However, substrate specificity of 14R and 16R did not correlate with anticoccidial efficacy since both were substrates for the enzyme from either E. tenella or E. acervulina.

Inhibition of Guanine Nucleotide Formation - Guanine nucleotide production in parasitic protozoa has been examined as a target in several parasitic protozoa. IMP dehydrogenase is responsible for the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP), from which



17 18

GMP is made. Inhibition of this enzyme using mycophenolic acid, 17, decreased guanine nucleotide synthesis in Leishmania tropica promastigotes.[44] It also eliminated 50% of the parasites at achievable peak serum levels when tested against amastigotes within macrophages. An attempt was made to inhibit guanine nucleotide production in Plasmodium falciparum grown in continuous erythrocyte culture.[45] Unlike the host mature erythrocyte, the parasite requires a continuous synthesis of guanine nucleotides in order to proliferate. Bredinin, 18R, inhibited the conversion of IMP to GMP in parasitized erythrocytes. This blockage was effective in controlling the parasite, arresting growth at the trophozoite stage. These studies demonstrate the importance of guanine nucleotide synthesis as a target in malaria.

Mycophenolic acid is effective at 0.5 ug/ml in completely inhibiting growth of E. tenella in tissue culture, although no activity in vivo is found even at very high doses, probably due to drug metabolism.[46] At the same concentrations that the drug is effective in tissue culture it also inhibits incorporation of labelled hypoxanthine into the guanine, but not adenine, nucleotide pools of the intracellular parasite. The drug effect is reversed by the addition of guanine to the media which subverts the block at the conversion of IMP to GMP by providing an alternate source of GMP through the very active parasite HGPRTase. Kinetic studies were carried out on the parasite enzyme and it was demonstrated to have an ordered bi-bi mechanism analogous to mammalian enzymes.[46] The inhibition patterns of 17 with the enzyme showed that it was adding to a complex of enzyme and product XMP, and a chemical basis for this very potent inhibition was proposed.

Thymidylate Synthetase-Dihydrofolate Reductase - Differences exist between the thymidylate synthetase, (TS), and dihydrofolate reductase, (DHFR), activities of protozoa compared to the enzyme from other sources. In species of Crithidia, Leishmania, Trypanosoma, Eimeria, Tetrahymena and Euglena it was demonstrated that the activities are encompassed in a bifunctional protein.[47] The apparent molecular weight of the bifunctional protein is close to the sum of the molecular weights of the separate enzymes from other sources. The Eimeria tenella enzyme was unusually large, even compared to other protozoa. Limited proteolysis with 5 different endopeptidases of the complex obtained from Leishmania tropica demonstrated rapid, time-dependent loss of TS activity with no effect on DHFR activity.[48] A 20000 MW fragment obtained from the proteolysis was homologous with the TS sequence of human, bacterial and viral enzymes. The TS-DHFR polypeptide consists of a DHFR sequence at the blocked N-terminus and TS sequence at the C-terminal end.

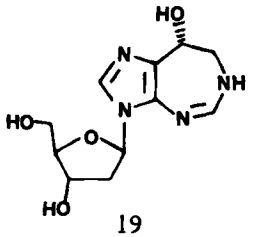
Leishmania tropica promastigotes highly resistant to methotrexate, MTX, were obtained by passaging the organisms in increasing concentrations of the drug.[49] The resistant organisms withstood 1 mM MTX concentrations and have a 40 fold increase in DHFR-TS. Amplified regions of DNA were visualized on stained gels of restriction digests. When propagated in drug free medium, the amplified DNA and DHFR-TS protein decreased to wild-type levels. However, rechallenging these revertants resulted in a more rapid development of resistance than for wild-type. The Leishmania gene amplification produced extra-chromosomal closed circular DNA in a manner similar to that found during gene amplification in mammalian cells.[50] The amplified DNA was in a 30 kilobase region which was cloned into vectors and utilized as a probe of leishmanial mRNA. Four mRNA species that were present in both wild type and drug resistant cells, were identified by hybridization although they were 40-fold more abundant in the resistant cells.[51]

Field isolates of Plasmodium falciparum have been shown to have large differences in sensitivity towards pyrimethamine[52] and in vitro induction of resistance to aminopterin has been demonstrated for this organism.[53] Synergistic

activity against P. falciparum has been demonstrated in vitro for pyrimethamine and sulfadoxine.[54] Kinetic and molecular properties have been studied for the DHFR from pyrimethamine sensitive and resistant strains of Plasmodium chabaudi.[55] The amount of enzyme present, the molecular weight, and specific activities were identical. However the K_m for dihydrofolate was 4-fold lower for the resistant parasite and the K_i was 100 fold higher for pyrimethamine. A Leishmania tropica mutant highly resistant to 10-propargyl-5,8-dideazafolate, an inhibitor of TS, has been obtained which has high levels of the bifunctional TS-DHFR and amplified DNA sequences.[56] No structural alteration in the protein appears to accompany drug resistance. The amplified DNA is similar to the 30 kilobase segment obtained in MTX resistant organisms.

Deoxynucleoside Phosphotransferase - Trichomonas vaginalis, a human pathogen, lacks the ability to synthesize purine and pyrimidine nucleotides de novo, and is also incapable of converting ribonucleotides to deoxyribonucleotides.[57] Therefore, a deoxynucleoside phosphotransferase activity found in the 100,000 xg pellet is required for DNA synthesis. The enzyme recognizes thymidine, deoxyadenosine, deoxyguanosine and deoxycytidine as acceptors, whereas TMP, dAMP, dGMP, dCMP, dUMP, 5-F-dUMP, and p-nitrophenylphosphate can serve as donors. It has a 200000 MW and K_m values of 2-3 mM for the deoxyribonucleosides, and may be a useful target for antitrichomonal therapy.

Adenosine Deaminase - Adenosine deaminase inhibition has been studied as a therapy for malaria. Adenosine deaminase activity is increased in Plasmodium falciparum infected erythrocytes compared to uninfected controls, and was demonstrated to be of parasite origin.[58] A single iv. bolus of the known inhibitor of adenosine deaminase, 2'-deoxycoformycin, 19, produced a dramatic reduction in parasitemia in P. knowlesi-infected rhesus



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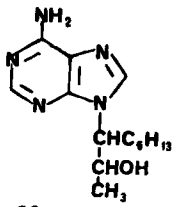

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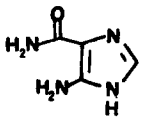

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monkeys, suggesting that the enzyme is a worthwhile therapeutic target.[59] Adenosine deaminase from P. lophurae and its host cell, the duck erythrocyte, were purified.[60] The crude lysate for infected erythrocytes had 60-fold more activity than uninfected cells. The two enzymes showed differing binding abilities towards an adenosine affinity column. Also, the duck erythrocyte enzyme was completely inhibited by 1.3 uM of erythro-9-(2-hydroxy-3-nonyl) adenine, (EHNA), 20, whereas no inhibition was found with the parasite enzyme. Interestingly, this same compound had, at higher concentrations, an effect on the motility of Leishmania promastigotes, apparently due to inhibition of axonemal dynein ATPase activity.[61] Erythrocytes deficient in adenosine deaminase were infected with P. falciparum and the resulting parasite adenosine deaminase could be studied without host background activity.[62] The enzyme was not cross reactive with antibody against human erythrocyte adenosine deaminase. Although it was inhibited by 19, it was also relatively insensitive to inhibition by 20, as was the

duck malaria enzyme.

GMP Reductase and Guanine Aminohydrolase - The enzyme GMP reductase from Leishmania donovani, which catalyzes the conversion of GMP + NADPH + H^+ to IMP_{63} + $NADP^+$ + NH_3, has been identified as a possible chemotherapeutic target.[63] The enzyme was highly purified from promastigotes and kinetic studies demonstrated inhibition by 1RP and by 13RP. These compounds were 100 and 20-fold, respectively, more potent as inhibitors of the parasite enzyme than they were for the corresponding enzyme from human erythrocytes. The enzyme guanine aminohydrolase from Leishmania spp. promastigotes was inhibited by 4-amino-5-imidazole carboxamide, 21, and this compound also inhibited the growth of these organisms, suggesting this enzyme as a target.[64]

Summary - It is clear from even this non-exhaustive survey of agents acting on purine and pyrimidine nucleotide metabolism in parasitic protzoa, that opportunities exist to find chemotherapeutic agents based upon an understanding of the differential metabolism of parasite and host. In many cases, drugs active in one organism have simply not been tried in others against which they may likely show activity.

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Chapter 24. DNA Topoisomerases as Therapeutic Targets in Cancer Chemotherapy

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Introduction - Significant progress has been made in the elucidation of the mechanisms of action of some anticancer drugs. However, there are still large numbers of anticancer drugs whose mechanisms of action are unknown. Even for well studied anticancer drugs, such as antimetabolites, alkylating agents and tubulin binders, the exact cell killing mechanism is still unclear. Recent findings have shown that a large number of clinically important anticancer drugs (e.g. anthracyclines, anthracenediones, acridines, actinomycins, ellipticines and epipodophyllotoxins) affect the breakage-rejoining reaction of mammalian DNA topoisomerase II by trapping a covalent enzyme-DNA cleavable complex. This phenomenon may provide new clues to the mechanism of cell killing by anticancer drugs. DNA topoisomerase II may be a key component in the regulation of chromatin conformation and cell proliferation. Further understanding of the roles of topoisomerase II in growth regulation may lead to more effective treatment for human neoplasms.

DNA Topoisomerases - DNA topoisomerases are enzymes which control the topology of DNA[1-4]. They are involved in DNA replication, RNA transcription and recombination. Based on their mechanism of action, DNA topoisomerases have been categorized into two types. Type I and type II DNA topoisomerases catalyze the topological changes in DNA by transiently breaking one strand or two strands of the DNA helix, respectively. The most characteristic reaction of a type I DNA topoisomerase is the relaxation of superhelical DNA. The type II topoisomerases can catalyze the passing of two DNA segments which can lead to a number of topoisomerization reactions of DNA such as supercoiling/relaxation, knotting/unknotting, and catenation/decatenation. In bacteria, the relaxation activity of topoisomerase I (omega protein) and the supercoiling activity of topoisomerase II (DNA gyrase) oppose each other and maintain the superhelical state of the bacterial chromosome in a dynamic balance. The superhelical state of the chromosomal DNA has an important regulatory role in DNA replication and gene expression. Although the roles of topoisomerases in bacterial growth regulation are still unclear, it is known that the superhelical density of a plasmid DNA is much higher in logarithmically growing cells than in stationary phase cells[5]. The exquisite sensitivity of ribosomal RNA synthesis to the superhelical state of DNA is another example of the importance of DNA tertiary structure on growth control. Very little is known about the roles of mammalian DNA topoisomerases in growth regulation. Recent studies, however, suggest that mammalian DNA topoisomerase II is highly regulated by the growth state of cells[6,7]. The antitumor activity of topoisomerase II -targeting anticancer drugs may well be determined at least in part by the differential regulation of topoisomerase II in neoplastic cells.

Protein-linked DNA Breaks Induced by a Number of Antitumor Drugs

Adriamycin, an anthracycline, has been one of the most important anticancer drugs in clinical use. Because of its strong intercalative mode of DNA binding, adriamycin interferes with many DNA functions such as replication and transcription[8,9]. It can also be reduced to a semiquinone radical which damages DNA and cell membranes[10]. The possibility that the membrane may be the cytotoxic target is further supported by the experiments which show that adriamycin can exert its effect without entering cells[11]. Despite these studies, the actual cell killing mechanism remains obscure. Using the alkaline elution technique, adriamycin and other intercalative antitumor drugs (anthracenediones, acridines, ellipticines and actinomycins) have been shown to induce DNA strand breaks and protein-DNA crosslinks in cultured mammalian cells[12-16]. The DNA strand breaks and protein-DNA crosslinks appeared synchronously and at a stochiometric ratio of approximately one[13]. These results suggested that adriamycin and other intercalative antitumor drugs may induce protein-linked DNA breaks in cells. Another unusual phenomenon of DNA damage induced by intercalative antitumor drugs is the rather rapid 'repair' of the damage upon drug removal[12-16]. The lack of correlation between protein-linked DNA breaks and drug cytotoxicity among structurally dissimilar drugs has led to the notion that protein-linked breaks may not be related to the cytotoxic action of these antitumor drugs[15]. It was thought that protein-linked breaks might be the consequence of a repair process rather than the result of direct damage to DNA. According to this view, the initial damaging event was the conformational change of DNA induced by drug intercalation. A repair nuclease or topoisomerase might thus be activated to counteract the torsional stress on chromosomal DNA. However, more recent studies have indicated that this interpretation is incorrect[17-19]. Protein-linked DNA breaks most likely reflect the direct DNA damage by intercalative as well as some nonintercalative antitumor drugs (see below). The ability of a drug to intercalate DNA does not correlate with its ability to induce protein-linked DNA breaks. Ethidium bromide which is a strong DNA intercalater does not induce protein-linked DNA breaks in cultured cells[18].

Evidence that DNA Topoisomerase II Is the Drug Target Responsible for Protein-linked DNA Breaks

The possibility that a topoisomerase may be involved in the action of intercalative antitumor drugs has been suggested from analyses of protein-linked DNA breaks in cultured mammalian cells. The successful synthesis of an acridine antitumor drug, m-AMSA, which produces large numbers of protein-linked breaks in cultured mammalian cells has made the analyses of the protein target easier[20-22].

The first clue came from the analyses of the ends of the broken DNA using exonucleases. It appeared that the 5' ends of the broken DNA were blocked by protein[23]. Since mammalian DNA topoisomerase II can be trapped as a covalent protein-DNA complex with the protein blocking the 5' ends of the broken DNA[24], this initial experiment has led to a number of extensive analyses of the possible involvement of topoisomerase II using both a purified system and cultured mammalian cells.

A. DNA Cleavage Reaction in a Purified System

The transient breakage of a DNA backbone bond by a topoisomerase is accompanied by the formation of a covalent enzyme-DNA intermediate[1-4]. Upon addition of a strong protein denaturant, the breakage of DNA occurs with the enzyme covalently attached to the

broken ends. The covalent linkage between the topoisomerase and DNA is always a tyrosyl phosphate bond for all DNA topoisomerases studied thus far. The polarity of the DNA strand which is covalently linked to the topoisomerase depends on the type of topoisomerase. All type II DNA topoisomerases are linked to the 5' phosphoryl ends of the broken DNA strands. Detailed studies of the cleavage reaction have shown that the double-strand breaks induced by type II DNA topoisomerases are staggered by four bases. The type II DNA topoisomerase (or the breakage-rejoining subunit of a type II DNA topoisomerase) is covalently linked to the 5' protruding phosphoryl end, while the 3' hydroxyl end is recessed by four bases. Most type I DNA topoisomerases are covalently linked to the 5' phosphoryl end of the broken DNA and the breaks are predominantly single-strand breaks. Different from most other topoisomerases but similar to the integrase of phage lambda, eukaryotic DNA topoisomerase I is covalently linked to the 3' phosphoryl end of the broken DNA strand. The significance of the polarity of DNA linkage is unclear.

In contrast to DNA gyrase, purified mammalian DNA topoisomerase II can cleave DNA rather efficiently in the absence of specific inhibitors[24]. Both single- and double-strand breaks are detected. Several experiments have strongly suggested that DNA cleavage by purified mammalian DNA topoisomerase II is due to the formation of a reversible enzyme-DNA complex, termed the cleavable complex. Irreversible DNA cleavage is observed when the cleavable complex is exposed to a protein denaturant. If prior to the addition of a protein-denaturant, a salt solution (e.g. 0.5 M NaCl) is added to the reaction, DNA cleavage is largely abolished. The possibility that the cleavable complex is related to the proposed transient intermediate is strengthened by the observation that the linking number of the DNA after high-salt treatment (a reversal experiment) was not detectably changed[24]. This result indicated that the two 'putative' broken DNA ends at the site of the cleavable complex were held tightly so that the relative rotation of the two broken DNA ends could not occur. This type of cleavable complex had been proposed to explain the strict linking number jump (in steps of two) during the relaxation reaction of topoisomerase II[25]. In contrast to the catalytic activity of topoisomerase II, the cleavage activity of topoisomerase II does not require ATP.

The first antitumor drug tested in the purified system was the synthetic acridine m-AMSA[19,26]. In the presence of m-AMSA, the DNA cleavage activity of mammalian topoisomerase II was greatly stimulated. Analyses of the cleavage reaction have shown that m-AMSA most likely interferes with the breakage and rejoining reaction of topoisomerase II by stabilizing a cleavable complex. Treatment of the cleavable complex with a protein denaturant results in DNA cleavage and the covalent linking of a topoisomerase subunit to each 5' phosphoryl end of the broken DNA strand. Both single- and double-strand DNA breaks were observed. The topoisomerase-mediated DNA cleavage induced by m-AMSA can be 'reversed'either by high salt or dilution treatment. It is likely that m-AMSA dissociates from the enzyme-DNA complex following dilution, and thereby reestablishes the normal equilibrium which favors the non-cleavable complex[19].

The successful demonstration that m-AMSA can induce topoisomerase-mediated DNA cleavage *in vitro* has led to the screening of a number of other intercalative as well as non-intercalative antitumor compounds. It was shown that both intercalative drugs (e.g.

ellipticine, adriamycin, mitoxantrone and actinomycin D) and non-intercalative epipodophyllotoxins (e.g. VP-16[27-31] and VM-26) similarly induced topoisomerase II-mediated DNA cleavage[27-31]. Different cleavage patterns have been observed for various drugs. Drugs of the same chemical class stimulated cleavage at similar sites, while drugs of a different chemical class showed strikingly different cleavage patterns[27]. The basis for the cleavage site specificity is not known. It is known, however, that type II DNA topoisomerases from different sources cleave DNA at different sites even when the same antitumor drug is used. Furthermore, different type II DNA topoisomerases have quite different sensitivity to these antitumor drugs. For example, both T4 phage-induced topoisomerase and calf thymus DNA topoisomerase II are very sensitive to antitumor drug m-AMSA[32]. *E. coli* DNA gyrase, on the other hand, is very resistant to m-AMSA[32].

B. Drug-induced DNA Cleavage on Cellular Chromatin in Cultured Mammalian Cells

Many lines of evidence have indicated that DNA topoisomerase II is responsible for drug-induced DNA cleavage on cellular chromatin in cultured mammalian cells. One of the most convincing experiments came from the analyses of the drug effect on SV40 cellular chromatin[33,33]. Treatment of SV40 infected monkey cells with m-AMSA or VM-26 resulted in both single- and double-strand breaks on SV40 viral chromatin. These breaks were shown to be covalently associated with protein based on their sensitivity to phenol extraction. Two pieces of evidence suggest that the covalently linked protein is DNA topoisomerase II. (a). Cleavage sites on intracellular SV40 DNA were strikingly similar to those produced on purified SV40 DNA by purified DNA topoisomerase II. (b). The protein-linked SV40 DNA was specifically immunoprecipitated by antisera against topoisomerase II. Further support came from a study using m-AMSA. A single DNA cleavage activity co-purified with the catalytic activity of calf thymus DNA topoisomerase II at all chromatographic steps of the enzyme purification[35]. Moreover, chromosomal DNA fragments from mouse cells exposed to m-AMSA have been demonstrated to contain a 175 kd terminal polypeptide[36]. These results strongly suggest that DNA topoisomerase II is the protein target responsible for DNA cleavage on cellular chromatin.

Mechanism of Cell Killing

As mentioned earlier, different classes of antitumor drugs induce distinct yet different DNA cleavage patterns. The drugs in the same chemical class, however, cleave DNA at identical sites. The structure-activity-relationship is most meaningful if antitumor drugs within the same chemical class are compared. The large number of synthetic acridines allowed such an analysis. The level of protein-linked DNA breaks induced by the acridine derivatives (both in the purified system and in cultured L1210 cells) correlated with drug cytotoxicity[36]. Other studies have also suggested that drug-induced cleavable complexes are responsible for cytotoxicity[18]. It is not clear, however, how the drug-induced formation of reversible cleavable complexes lead to rapid cell death.

The striking analogy to the bactericidal effect of nalidixic acid gives clues to the possible mechanism of cell killing by topoisomerase II-targeting drugs. Treatment of exponentially growing cultures of *E. coli* with nalidixic acid causes immediate but reversible arrest of DNA synthesis. Irreversible cell kill occurs after a lag time[37,38]. Inhibitors of RNA or protein synthesis inhibit

irreversible cell kill by nalidixic acid[39]. Genetic studies have shown that nalidixic acid is a strong inducer of SOS repair functions which are the inducible cellular responses to the distress signals[40]. Interestingly, E. coli mutants which are unable to induce SOS functions are more resistant to the bactericidal effect of nalidixic acid. These results suggest that at least some of the SOS repair functions may be critically involved in the bactericidal action of nalidixic acid. This unusual requirement of SOS functions for the rapid cell killing is also observed during thymineless death[41]. The significance of the involvement of SOS functions in the rapid cell killing process is not fully understood. Studies have also shown that mutations affecting the Rec BC protein block the induction of Rec A mRNA by nalidixic acid[42]. It seems possible that Rec BC nuclease may be involved in the induction of SOS functions by nalidixic acid.

The formation of drug-induced cleavable complexes in mammalian cells may induce rapid cell death by a similar mechanism. However, little is known about the SOS-like repair functions in mammalian cells. It seems possible that the high frequency of sister-chromatid exchanges, chromosomal aberrations, and rapid cell death may all be due to the cellular response to drug-induced cleavable complexes. Understanding the cellular responses to the drug-induced cleavable complexes in mammalian cells is likely to shed light on the mechanism of cell killing.

SUMMARY - There have been increasing evidence that DNA topoisomerase II is the therapeutic target of many intercalative antitumor drugs and non-intercalative epipodophyllotoxins (VP-16 and VM-26). These drugs appear to affect the breakage-reunion reaction of topoisomerase II by trapping an enzyme-DNA cleavable complex. Although the chemical nature of the cleavable complex is still unclear, exposure of the cleavable complex to strong protein denaturants such as sodium dodecyl sulfate results in DNA strand breakage and covalent attachment of one topoisomerase II subunit to each 5' phosphoryl end of the broken DNA strand via a tyrosyl phosphate bond. Much has to be done about the chemistry of drug-enzyme-DNA interactions. The biological consequences of the drug-induced cleavable complexes may involve rapid cell death, increased frequency of sister-chromatid exhanges, and chromosomal aberrations. The possible involvement of inducible repair functions has been suggested from studies of the bactericidal action of nalidixic acid. Recent studies have also suggested that DNA topoisomerase I is a potential target in cancer chemotherapy[43]. It seems possible that all topoisomerases may be potential targets in either antimicrobial or antitumor chemotherapy.

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Chapter 25. COLONY STIMULATING FACTORS

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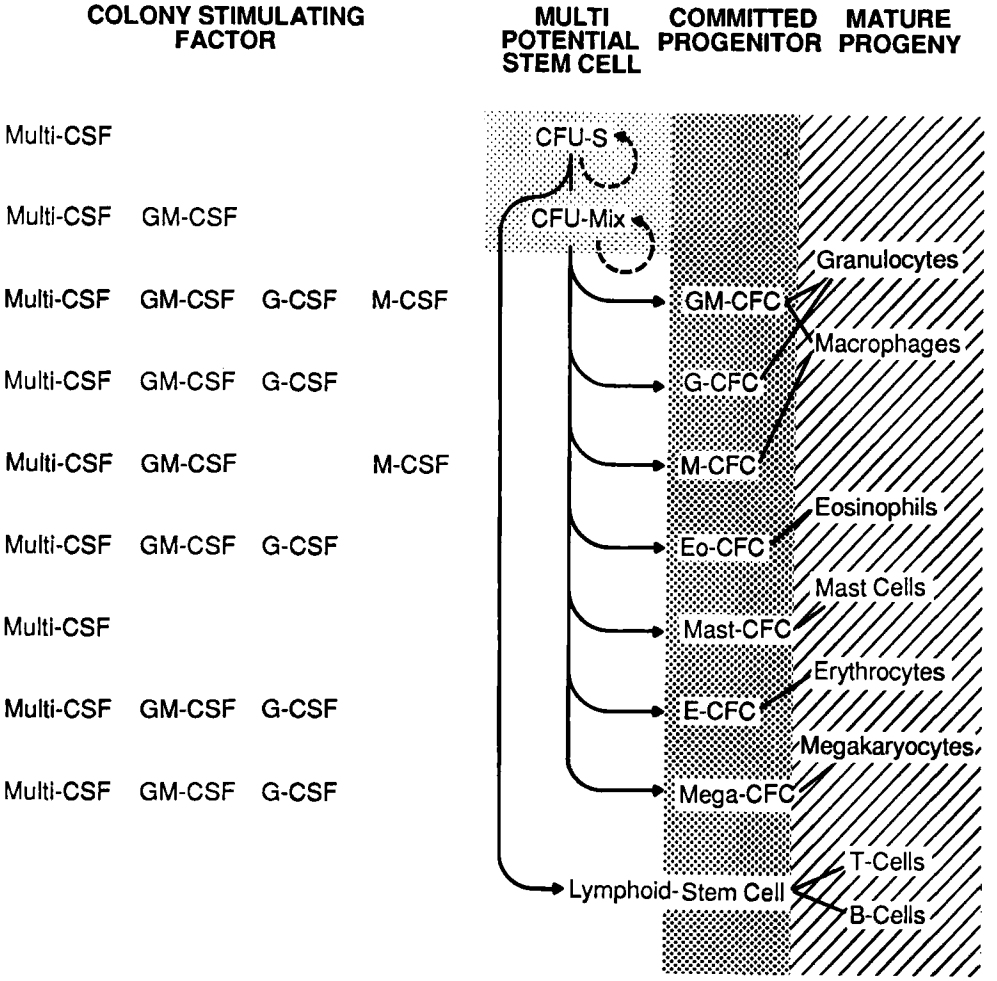
Introduction - The inoculation of mouse bone marrow cells into lethally irradiated and genetically compatible recipients results in the formation of spleen nodules containing erythroid, myeloid and megakaryocytic cells.[1] Cytogenetic studies established the clonality of these hemopoietic spleen colonies and proved that the colony forming unit-spleen (CFU-S) is also the precursor cell for the lymphoid lineage.[2] Some of the CFU-S were capable of dividing to give rise to daughter CFU-S, a process designated as self renewal.[3] They were regarded as multipotential stem cells of the hemopoietic system in that they both self renew and give rise to differentiated cells of all lineages.

Histological and kinetic studies performed in mice and other species provided insight into the nature of events occuring in the bone marrow during differentiation of these stem cells into mature progeny.[4,5] Nevertheless, the study of stem cells in an *in vivo* system had obvious limitations which created the need to study hemopoiesis under controlled *in vitro* conditions. Clonal growth of blood cells *in vitro* was achieved by seeding bone marrow cells embedded in agar medium supplemented with serum onto a "feeder layer" of mouse embryo fibroblasts Following a week of incubation hemopoietic colonies developed. Feeder cell layers could be replaced by media conditioned by embryo fibroblasts or extracts of a variety of other cells and tissues.[6,7] The biologically active substance(s) present in those conditioned media termed "colony stimulating factor" (CSF), induced the formation of colonies containing granulocytes and macrophages but not cells of other hemopoietic lineages. One of the immediate questions raised by these observations was whether the cells forming colonies *in vitro* (colony forming cell-culture, CFC) were identical to or different from the hemopoietic stem cell (CFU-S). A variety of experimental approaches to this and related questions lead to the realization that the hemopoietic system is composed of a hierarchy of cells (Figure 1). The pluripotent stem cell gives rise to a number of independent progenitor cells, committed to the various differentiation lineages and capable of forming colonies *in vitro*. Moreover, the so-called colony stimulating factor was one of a family of glycoprotein molecules having different although overlapping target cell specificities. The clonal growth and differentiation of the various types of CFC's strictly depended upon the presence of an appropriate CSF. Many of these stimulating factors have been purified and molecularly cloned.

The functions of colony stimulating factors have been reviewed in great detail by Metcalf.[8,9,10] The purpose of this review is to briefly summarize and compare the properties of these molecules, describe newly discovered hemopoietic factors, and evaluate the *in vivo* roles and potential medical uses of CSF's.

Figure 1 depicts the hierarchy of cell types in the blood system. It also indicates the types of colony stimulating factors that have been reported to affect hemopoietic growth, differentiation or functionality (Factors affecting lymphoid cells such as BSF-1, BCGF, BCDF and IL-2 as well as erythropoietin will not be discussed in detail). It is evident that much overlap exists in target cell specificities. Nevertheless, most of the colony stimulating factors have been named on the basis of their first described function, which is, in many cases, also their most pronounced bioactivity.



LEGEND TO FIGURE 1: Target cells for colony stimulating factors in the hemopoietic system. The multipotential hemopoietic stem cell compartment which is endowed with a capacity for self renewal (broken line arrows) gives rise to a variety of committed progenitor cells. The right part of the figure shows mature progeny derived from each progenitor cell type. The colony stimulating factors indicated on the left part of the figure are horizontally aligned with the various target cells that they have been shown to act on.

Multipotential Colony Stimulating Factors - A number of murine multipotential CSF's designated Multi-CSF,[11] IL-3,[12] HCGF,[13] MCGF,[14] PSF,[15] SAF,[16] BPA[17] and CSF-2α[18] are all now known to be the same molecular species. It is a glycoprotein with a molecular weight of 23-30 kD (the variation in molecular weight of this and other CSF's is due to heterogeneity in glycosylation). The nonglycosylated protein is about 16 kD and contains four cysteine residues. The factor is produced by mitogen stimulated lymphocytes and by a myelomonocytic cell line (WEHI-3B). cDNA clones for this factor were obtained from myelomonocytic leukemia cells[19] and from a concanavalin A activated mouse T cell clone.[20-22] The cloning strategy in the latter case was based upon the availability of cell lines such as the MC/9 mast cell line, that strictly requires Multi-CSF for its survival in culture. mRNA from the activated T cell clone was first selected by size fractionation. It was then used to construct a cDNA library in the pcD vector of Okayama and Berg, which allows expression in mammalian cells.[23] The cDNA library was further screened by hybrid selection. One subpool that consistently yielded mast cell growth factor activity in injected oocytes was further subdivided and reexamined. Transfection of COS-7 monkey cells by some of the selected pcD clones resulted in expression of functional MCGF. This recombinant factor was eventually found to be identical to IL-3.[20-22] The gene for this CSF was located on chromosome 11.[24] It is present as a single copy and has five exons.[25-27] L cells transformed by a plasmid carrying this gene (p λ MGM12-4) constitutively expressed MCGF activity, confirming the functional integrity of the cloned gene.[26]

Multi-CSF is characterized primarily by its capacity to affect the proliferation of CFU-S and induce the differentiation of Multi-CFC (an additional subpopulation of stem cells, see Figure 1). It can induce colonies containing myeloid, erythroid as well as megakaryocytic cells.[19] In addition to these activities Multi-CSF affects both committed and mature cells and is required for *in vitro* survival and proliferation of mast and myeloid cell lines.[28] Cell surface receptors for Multi-CSF were identified on cell lines dependent upon this factor for their growth.[29] Only unlabeled Multi-CSF was able to compete with ^{125}I-labeled Multi-CSF for binding. The molecular weight of the receptor was estimated to be greater than 72 kD.[18]

A valid human analogue to IL-3 has not been reported thus far. However a different factor termed pluripoietin has been purified from the human bladder carcinoma 5637 cell line. This factor has a molecular weight of 18 kD. It stimulates the growth of mixed, granulocyte/macrophage (GM) and erythroid colonies, and induces the differentiation of the promyelocytic leukemia cell line HL-60.[30]

Colony stimulating factors for granulocytes and macrophages - GM-CSF is a glycoprotein having a molecular weight of 23 kD. In contrast to Multi-CSF which is not found *in vivo* at detectable levels and is secreted only by stimulated lymphocytes, GM-CSF is synthesized and can be extracted from a variety of cells and tissues (Reviewed in [9,10]). The first GM-CSF to be purified to homogeneity was obtained from mouse lung. The molecular weight of this GM-CSF protein is 14 kD.[31] Like Multi-CSF the protein contains 4 cysteine residues. Oligonucleotide probes prepared on the basis of NH_2-terminal amino acid sequence, were used to search for relevant clones in a library constructed from mRNA of producer lung tissue.[32-33] This method yielded cDNA clones that could select mRNA which encoded functional GM-CSF after inoculation

onto ooycytes. Since this cDNA was not full length other methods were used. Screening of cDNA libraries by expression of transfected COS-7 monkey cells enabled direct isolation of cDNA clones encoding biologically functional GM-CSF. Based upon homology of cDNA sequences, both the mouse and the human genes were isolated.[34] The mouse gene for GM-CSF which has 4 exons, is present as a single copy and is localized on chromosome 11.[32] Recombinant GM-CSF has been expressed in a fully active form in mammalian, yeast and bacterial cells.[34-37] The study of the GM-CSF gene and the structure of a cDNA clone containing a complete copy of the mRNA for this factor indicated that there were two possible forms of the molecule.[31,38] Two initiation codons were detected in the mRNA. Whereas the second AUG initiated translation of a secreted factor, the first AUG was speculated to initiate the translation of an integral membrane protein.

The target cells for murine GM-CSF are granulocytes and macrophages. However, both the natural and recombinant factors also induce eosinophil, megakaryocyte and erythroid colony formation, when included in the growth medium at high concentration, and have moderate effects on the growth of stem cells.[11,39,40] High and low affinity receptors for murine GM-CSF were detected using ^{125}I-labeled ligand.[41] Similarly, high affinity receptors for native and recombinant human GM-CSF (also termed CSF-α) were detected on the responsive KG-1 and HL-60 human cells but were absent on non responsive cells.[18]

Colony stimulating factors for granulocytes - G-CSF is predominantly an inducer of granulocyte differentiation although weak effects on erythroid and multipotential cells have been reported.[42] As with GM-CSF, it is found in a variety of tissues. Murine G-CSF has been purified to homogeneity and found to be a glycoprotein of 25 kD.[43] cDNA for this murine factor has not as yet been reported. In addition to its capacity to induce the formation of granulocyte colonies, G-CSF is also a potent inducer of terminal differentiation of myelomonocytic leukemia cell lines.[43,44] The latter property is shared by a different factor termed MGI-2. Purified G-CSF was iodinated without loss of bioactivity. ^{125}I-labeled G-CSF bound to the myelomonocytic leukemia cell line WEHI-3B, to the J774 macrophage tumor cell line and to murine bone marrow cells. No binding to other tumor cell lines or to murine thymocytes was observed.[45] A human analog of mouse G-CSF termed CSF-β was demonstrated in conditioned media from the human bladder cell line 5637 by separation from CSF-α using phenylsepharose fractionation.[46] The human G-CSF had all the properties described for the murine factor. More importantly, the human and the mouse molecules had almost complete receptor binding- cross reactivity.[46] Recently cDNA for a human G-CSF has been isolated from a human squamous carcinoma cell line (CHU-2). The latter produced high titers of colony stimulating activity, inducing the formation of colonies containing almost exclusively, granulocytes.[47] This CSF was purified to homogeneity and partial amino acid sequence was obtained. Synthetic oligonucleotide probes were then used to screen cDNA libraries. Two cDNA clones obtained in this manner were expressed in COS cells and exhibited G-CSF activity.[47] These cDNA's encoded two different protein species differing in one position where three amino acids were deleted/inserted. The cDNA was used to screen a human gene library and clone the gene for G-CSF. The expression of the G-CSF gene in COS monkey cells indicated the existence of two mRNA's as shown by S1 analysis.[48] It is not known whether this recombinant human factor has a differentiation inducing capacity for myeloid leukemia cells or how it is related to CSF-β.

Colony stimulating factors for macrophages - Alternative designations used for macrophage stimulating factors are M-CSF[9] and CSF-1.[49] The murine M-CSF is a glycoprotein of 70 kD. A considerable part of this molecule consists of polysaccharide. The protein part is composed of two identical 14kD subunits. This murine CSF has not been cloned despite the fact that it has been purified to homogeneity.[50-52] The major source for purification of M-CSF was mouse L-cells. It is produced by fibroblasts from various sources, stromal cells from the bone marrow and is found in many tissue extracts including embryonic tissue.[53-55] The target cell range for M-CSF is rather limited and its most obvious targets are macrophages. It is, however, capable of inducing the differentiation of small numbers of granulocytes, when it is applied to murine bone marrow cells. M-CSF is required for both the survival and proliferation of macrophages.[56,57] As in the case of Multi-CSF and GM-CSF, factor dependent cell lines have been derived that require M-CSF for their in vitro growth.[58] The availability of both iodinated M-CSF and antibodies to the factor led to the development of a specific radioimmunoassay. Preparations containing M-CSF are used to compete for the reaction between purified iodinated M-CSF and its antibody.[59] ^{125}I-labeled M-CSF was also useful in detection of specific membrane receptors on cells of the macrophage lineage.[60,61] The receptor is a protein of 165 kD and is related to the c-fms oncogene product.[62] A human analogue for the murine M-CSF has been molecularly cloned from the MIA-PaCa pancreatic carcinoma cell line.[63]

MGI-1 and MGI-2 - Certain myeloid leukemia cell lines that are independent with respect to their capacity to proliferate in culture have been shown to terminally differentiate and stop growing upon the addition of a factor designated MGI-2.[64-68] In normal cells, MGI-2 is endogenously produced following induction of growth by colony stimulating factors. These latter factors are referred to by the collective name MGI-1. Colony stimulating factors are, therefore, suggested to be primarily proliferation inducers. In contrast MGI-2, which has a novel capacity to bind to double stranded DNA, is a differentiation inducer.

Newly identified factors with activities overlapping those of CSF's - Recently, two factors with a multipotential target cell range have been described. Since both types are tentatively designated IL-4, we shall refer to them by their target cell range. A product of an activated T cell line was identified, that had both T cell growth factor (TCGF) and mast cell growth factor (MCGF) actvities but appeared to be distinct from both IL-2 and IL-3 as indicated by biochemical fractionation.[69] A cDNA clone for this factor was obtained by construction of a cDNA library in the pcD vector and screening by transfection of COS-7 monkey cells.[70] The recombinant factor stimulated T cell and mast cell growth, induced Ia in B cells and enhanced IgG_1 and IgE secretion by B cells. The latter activities are indistinguishable from those of the B cell stimulating factor BSF-1.[70] An apparently identical cDNA clone has been obtained by others.[71] A second factor designated IL-4 has recently been reported.[72] It is a differentiation factor for eosinophils but has activities in common with B cell growth factor II (BCGF II). In this latter case no molecular cloning evidence is available to confirm the association between the two bioactivities.

Role of CSF's in the regulation of hemopoiesis - In the hemopoietic system, the stem cell pool represents a relatively minor population in

terms of cell numbers and is maintained intact throughout the entire life span due to the capacity of stem cells to self renew. The probability of divisions of stem cells that produce daughter stem cells is, at all times, equal to or larger than 0.5.[10] This prevents excess differentiation and depletion of the stem pool. The question has been raised as to whether CSF's are capable of mediating stem cell renewal. Are colony stimulating factors in such an event both inducers and attenuators of differentiation? As indicated above, IL-3 and GM-CSF have been shown to induce the proliferation of stem cells in culture. It is, however, evident that all of those effects are transitory and are followed by terminal differentiation. The only *in vitro* system that partially mimics self renewal phenomena that occur *in vivo* is the long term marrow culture in which hemopoiesis is sustained by stromal cells isolated from the hemopoietic microenvironment.[73-75] In these cultures, stem cell maintenance does not correlate well with the titer of CSF's. Multi CSF has not been detected in long term bone marrow cultures, and other subtypes were barely detectable. In contrast, primary stromal cell cultures and a variety of stromal cell lines derived from mouse bone marrow do produce colony stimulating activity which does not correlate with the cells ability to promote hemopoiesis [55,76-87] Moreover, stromal cells were shown to antagonize the activity of CSF's.[76] Both the maintenance of stem cells and their differentiation in the presence of stromal cells from the bone marrow appear to depend upon close proximity of the interacting cells. The factors operating there may be cell bound or acting over a very short distance.[76-87] Some are apparently unrelated to CSF's while others may be cell bound CSF species.

The most pronounced activity of the various colony stimulating factors is their ability to induce accumulation of large numbers of terminally differentiated and highly potent progeny.[88-90] It is anticipated that the role of CSF's *in vivo* will be mainly to expand the mature cell pool and populate the hemopoietic organs and peripheral blood. Bacterial infections, immune responses and other conditions which are accompanied by a demand for a large number of functional hemopoietic cells are also characterized by an increase in titer of CSF.[10] There are conflicting findings regarding the different subtypes of CSF's and their various *in vivo* roles.[91-93] The most pronounced effect follows administration of recombinant IL-3 by use of osmotic pumps. A marked enhancement of hemopoiesis occurred in both normal and irradiated mice.[94] The increase in stem cells occurred in extra medullary sites while the marrow population was decreased. GM-CSF has been reported to increase organ cellularity and content of CSC.[9] In contrast, M-CSF was not effective *in vivo*[95] and some granulocytic responses may be mediated via a mechanism that is not directly related to CSF.[96] There is a clear need to use the recently available recombinant CSF's for detailed studies of *in vivo* functions.[9]

Relationship to cancer and oncogenes - Many authors have proposed a relationship between CSF's or their receptors and cancer. This results from CSF's role in the proliferation of various haematopoietic cell lineages as well as its potential role in an autocrine model wherein cells would both express CSF receptors and produce CSF's. It has been postulated that CSF's or CSF receptors may represent the products of oncogenes. No homology has as yet been found between oncogenes and any of the CSF's. However, it has been well documented that the translation product of *c-fms* has a high degree of homology with the M-CSF (CSF-1) receptor.[62] v-fms is the viral oncogene of the McDonough strain of feline sarcoma virus.[62] Conceivably, as other CSF

receptors become characterized, homology may be shown with additional oncogene products. An additional association between the proto-oncogenes, FMS and GM-CSF genes and myeloid disorders was reported.[97] Both genes were localized to human chromosome 5 and are frequently deleted in neoplastic myeloid disorders.

A number of biological observations have provided evidence that myeloid leukemias are, in some instances, related to CSF's either in an autocrine fashion or by CSF's supplied extrinsically (reviewed in [9,10]). Primary myeloid leukemias in mice and man are often totally dependent on exogenous CSF for both survival and proliferation in vitro and this continues throughout the disease. Although the requirement for extrinsic CSF's would argue against an autocrine model for myeloid leukemias,[98] CSF's are produced in many normal tissues and it may not be easy to determine whether early leukemia cells are also producing CSF's.

Leukemia cells have frequently been shown to produce CSF's but it is not clear if these cells produce either abnormal amounts of CSF or abnormal forms of CSF (reviewed in [9]).

Potential clinical utility - The major potential clinical utility of CSF's is in the stimulation of haematopoietic tissue. This may be of particular importance in vivo in bone marrow transplantation or in bone marrow reconstitution concomitant with radiation therapy. Thus, CSF's may be useful in treatment of the side effects of radiation and cancer chemotherapy or in treating the side effects of radiation toxicity following accidental exposure (reviewed in [8,9,10]). In certain bone marrow transplants, it may be desirable to retain the greatest proportion of stem cells until after transplantation or reconstitution. In this case, CSF antagonists would have utility until after transplant when further development and differentiation of the various cell lineages is required.

The single clinical study reported using CSF purified from human urine, showed accelerated recovery of leukocyte levels in leukopenic patients.[99] This has obvious potential in the infectious disease area through stimulation of normal host defense mechanisms. The threat of infection is a major concern in leukopenia, and CSF therapy offers potential in non-specific stimulation of host resistance to infection (reviewed in [8,9,10]). More speculative are the potential usefulness of CSF's in general anemia, agranulocytosis or thrombocytopenia.

The possibilities for use of CSF's apply not only in vivo but also ex vivo. In the ex vivo mode a major opportunity is in the use of CSF's for production of appropriate stem cells and their mature progeny in marrow to be used for transplantation and/or reconstitution. Thus, marrow would be removed from a patient, maintained with "appropriate" CSF's in vitro while the patient was undergoing radiation therapy or cytotoxic chemotherapy and then used for subsequent reconstitution of the patient.

An interesting proposition is that CSF's may have utility in suppressing stem cell leukemias by driving the stem cells to differentiation into mature granulocytes and macrophages with concomitant suppression of stem cell self regeneration Indeed, G-CSF has been shown to suppress mouse myeloid leukemia cells by suppression of stem cell regeneration and induction of terminal differentiation.[44]

Since human myeloid leukemias depend on CSF for proliferation, it can be reasoned that suppression of CSF levels might result in suppression of the leukemia.[9] However, normal differentiation and development of granulocyte and macrophage lineages also require stimulation by CSF (see above).

The availability of recombinant CSF's and CSF receptor genes for both mouse and human systems will now permit definition of the role of CSF's under normal conditions as well as in various disease situations.

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Chapter 26. Atrial Natriuretic Factor

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Introduction - The role of the heart in the control of extracellular fluid volume through neural mechanisms has been well established for many years. However, investigators also postulated that the heart may influence renal functions through the release of humoral substances. Indeed, dense core granules, resembling secretory granules, were observed in the atria of mammalian hearts.[1] The granules were in close association with the golgi complex and contained a protein material. However, the natriuretic properties of the atrial material were not discovered until 15 years later. In 1981, deBold and co-workers demonstrated that intravenous injection of atrial, but not ventricular, extracts from rat hearts into anesthetized rats caused a marked but short-lived natriuresis and diuresis and lowered arterial pressure. The unknown material was referred to as atrial natriuretic factor (ANF).[3-9]

Since the initial reports, ANF research has progressed at an explosive rate. Within the last 5 years, the substance was isolated, purified and sequenced; the DNA sequence of the precursor peptide was discovered and receptor sites were identified; the pharmacologic actions of the natriuretic peptide were examined; assays for measurement of ANF were devised, and the clinical effects of ANF were reported. With the large amount of data available, it is beyond the scope of this review to present a detailed summary of all the biology of ANF. Numerous other reviews are available for additional information.[3-9]

Peptide sequence and gene isolation - Early attempts to isolate and purify active ANF peptides from mammalian atria resulted in the identification of several biologically active residues of diverse amino acid composition.[10,11] Subsequent purification and amino acid analysis of these residues revealed several low molecular weight (M_r) peptides containing an identical core sequence (as illustrated by the human sequence in figure 1) but varying in overall length, the longest peptide being 33 amino acids.[12-18]

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      95                  100                 105                 110
H-Leu-Ala-Gly-Pro-Arg-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met
              115                 120                 125
Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH
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1.

The various names and amino acid sequences of these early peptides are as follows: cardionatrin I, Ser 99-Tyr 126; atriopeptin I, Ser 103-Ser 123; atriopeptin II, Ser 103-Arg 125; atriopeptin III, Ser 103-Tyr 126; auriculin A, Arg 102-Arg 125; auriculin B, Arg 102-Tyr 126; ANF 1-33, Leu 94-Tyr 126; ANF 2-33, Ala 95-Tyr 126; ANF 3-33, Gly 96-Tyr 126; ANF 8-33, Arg 101-Tyr 126 and finally, α-human ANF, Ser 99-Tyr 126.

The natriuretic and vasoactive properties of these isolated peptides are similar to those of crude atrial extracts. As confirmation of the biological activity many of these peptides have been prepared synthetically.[15,18,19] In addition, the isolation and sequencing of six low M_r peptides from rat atria has been reported; some of these are identical to the above sequences.[20] Also reported were a rat 48 amino acid peptide of 5000 daltons[21] and the 126 amino acid pro-segment.[22] Additional high M_r rat sequences are also described.[23,24]

The successful isolation and cloning of complementary DNA (cDNA) sequences encoding ANF has revealed that active ANF is synthetized as a 152 amino acid precursor in rodents[20,25-27] and as a 151 amino acid precursor in humans.[27-29] These precursors are composed of a 126 amino acid pro-segment and a "leader" or "signal" sequence (pre-segment) consisting of 24 amino acid residues in the rat[20,27] and 25 amino acids in the human.[27-29] The signal peptide contains a large number of hydrophobic amino acids ending with alanine.[20,25,28] The pro-segment ANF peptide is thus derived from the prepropeptide by enzymatic cleavage at this site.[20,27] All the rat ANF segments reported above are contained in the C-terminus of the precursor peptide and they all contain a macro-ring structure consisting of 17 amino acids formed by a Cys 105-Cys 121 disulfide bridge.[20,27]

In contrast to the numerous peptides identified in the rat atria, only three distinct natriuretic factors have been identified in humans; α-hANF (28 amino acids),[6,29] β-hANF (56 amino acids)[21,29] and γ-hANF (126 amino acids).[29] Hence, γ-hANF is derived directly from the 151-residue precursor by removal of the 25 amino acid signal.[29] In contrast, β-hANF is thought to be an antiparallel dimer of α-hANF (28 amino residues). β-hANF, although lacking the specific ring structure observed in α-hANF, exerts appreciable natriuretic and hypotensive activity after intravenous injection in rats.[29] A diuretic/natriuretic response is also noted with γ-hANF; however, the β-peptide appears to have a slower onset and a somewhat longer duration of action compared to both the α- and γ-hANF. The N-terminal sequence of γ-hANF is closely homologous to the N-terminal of cardiodilatin, a 30-residue peptide isolated from porcine atrial tissue.[30]

Translation of the signal peptide occurs at the ATG codon at nucleotide positions 88-90, the first methionine codon in the message sequence. The signal sequence in the rat differs from that in the human by lacking a threonine at nucleotide positions 106-108. This codon is completely lacking in the rat pre-segment peptide. The sequence encoding the corresponding pro-segment (126 amino acids) immediately follows the 24 amino acid signal peptide and ends at residue 126, a tyrosine that is connected to the extra C-terminal Arg-Arg sequence. In the precursor hANF, translational termination occurs just after the tyrosine residue whereas in the rat precursor peptide, translation continues two amino acids beyond tyrosine 126 and ends at the other termination triplet, TAA. However, the absence of peptides that terminate in the Arg-Arg sequence in rat atrial extracts suggests that the C-terminal Arg-Arg is removed from the 152-residue precursor at an earlier stage to produce γ-rANF. In contrast to the hANF precursor peptide, the rat precursor contains a possible glycosylation site.[20,25]

Remarkable homology exists between both the amino acid and

nucleotide sequences of the human and rat pro-segments.[20] Conversely, the homology is considerably reduced in rat and human signal peptides. The 5'- and 3'-untranslated regions are, however, very similar.

Identification of the gene sequence of ANF has also peaked interest in the production of the atrial peptides by genetic engineering. Most commercially available ANF is currently synthesized by solid phase techniques, which are rather expensive. Genetic expression of the material may be less expensive and may make the therapeutic use of ANF more economically feasible.

Receptor localization and second messengers - Specific membrane receptors for ANF have been described in rat[35-39], rabbit[35,39], bovine[37] and guinea pig[39] vascular smooth muscle cells, in bovine endothelial cells[37], in rat and guinea pig kidney cortex[35,38-41], in rat and bovine adrenal cortex[38,39,41] and in rat adrenal medulla, liver, lung, intestine, heart, brain and eye.[38,39,42] Specific ANF receptors have also been reported in human kidney postmortem tissue[43] and cultured mesenchymal non-myocardial cells from rat heart.[44] Tissue ANF receptors from spontaneously hypertensive rats did not differ from those in normotensive rats.[39] The widespread distribution of ^{125}I-ANF binding sites suggests multiple activities for this peptide.

ANF increases cyclic guanosine monophosphate (cGMP) levels in rat kidney slices[45] and in primary cultures of renal tubular[45] and mesangial cells.[4] The latter cells are reported to be the principal target for ANF binding in the glomerulus.[4] ^{125}I-ANF also elevates plasma and urine cGMP levels in rats[38,45] largely as a result of selective activation of particulate guanylate cyclase in the glomeruli.[38] The presence of specific ANF binding sites on vascular smooth muscle is consistent with the finding that ANF produces relaxation of precontracted vascular strips by a direct effect.[35] Rat and human ANF exhibit similar binding characteristics in vascular smooth muscle. ANF inhibits adenylate cyclase activity[46] and stimulates intracellular cGMP formation in vascular smooth muscle cells.[36,37,47,48] As in the kidney, ANF also appears to activate particulate guanylate cyclase activity in these muscle cells.[47] The 17 member ring structure is essential for binding and cGMP formation,[48] and, ultimately, for vasorelaxation. The hydrophobic amino acid residue in position 110 is also important for receptor interaction and biological activity.[48] Exposure of vascular receptors to unlabeled ANF reduces Bmax with no change in K_D,[48] raising concerns regarding tolerance.

Recent attempts to isolate the ANF receptor have resulted in the identification of a protein band with an apparent M_r of 124 kDal in bovine adrenal and 130-140 kDal in rat adrenal[49] and kidney cortex membranes. Specific ANF receptors have also been identified and solubilized from glomeruli of rat kidney[40] and bovine adrenal cortex. Reports have been issued describing specific ^{125}I-ANF binding to proteins of 66 kDal and 180 kDal in cultured bovine aorta smooth muscle and endothelial cells, respectively.[50]

Renal pharmacology - On a molar basis, ANF is the most potent diuretic agent yet discovered. In conscious rats, intravenous infusion of ANF at doses as low as 25-50 pmol/min (75-125 ng/min) increases urine volume and the fractional excretion of sodium.[51] Similarly, intrarenal infusions of 4.8 pmol/kg/min represent threshold natriuretic doses in anesthetized dogs.[52] Administration of ANF by bolus injection or

continuous infusion immediately increases urine volume which is accompanied by enhanced urinary excretion of sodium, potassium, calcium, magnesium, chloride and phosphate.[4,8] Again, rat and human ANF elicit similar diuretic responses in dogs and rats. Based on maximal diuretic effectiveness, ANF most closely resembles the thiazide diuretics. However, loop diuretics (furosemide, etc.) are much more effective in stimulating maximal renal excretion of salt and water than ANF.[8]

The duration of action of the atrial peptides is greatly dependent on the mode of administration. Intravenous bolus administration of ANF produces only a short-lived natriuresis (10-40 min), due to rapid elimination from the plasma.[4,6-8] However, continuous infusion of the peptide for at least one hour yields sustained natriuretic and diuretic responses in conscious and anesthetized dogs for the duration of the infusion.[53-55] Oral activity of ANF is negligible.

ANF appears to act through a novel mechanism. Unlike other diuretic agents that inhibit renal tubular reabsorption of ions, ANF has no significant direct effect on tubular function. No ANF-specific receptor sites can be identified on the renal tubules[5] and ANF fails to alter salt or water reabsorption from isolated perfused renal tubule segments.[4,56] Although inhibition of sodium-phosphate co-transport is observed in proximal tubule brush border membrane vesicles prepared from rats treated with ANF prior to sacrifice, these effects are not observed when ANF is added to the membrane vesicles *in vitro*, suggesting that the inhibition of sodium-phosphate co-transport *in vivo* may not involve a direct tubular effect of the atrial peptides.[57,58] Hence, the *in vivo* inhibition may result from selective effects of ANF at other sites in the kidney.

The diuretic actions of ANF appear to be mediated primarily through alterations in renal hemodynamics. For example, the diuretic effects of ANF are very sensitive to changes in renal perfusion pressure. Modest reductions in renal perfusion pressure attenuate the renal responses to the atrial peptides, while more severe reductions in perfusion pressure completely antagonize the diuretic actions of ANF.[66] Conversely, acute increases in renal perfusion pressure markedly enhance the renal effects of the atrial peptides.

Rather than increasing total renal blood flow, ANF may alter intrarenal vascular resistance at two specific sites, the glomerulus and the papillary vasa recta. ANF binding sites have been identified in these tissues.[38-40] Of these two sites, the effects of the atrial peptides on the glomerulus are best described. In many[18,53-55,59-61] but not all[52,62,63] experiments, marked increases in glomerular filtration rate (GFR) are observed after ANF administration. The peptides enhance GFR through differential effects on afferent and efferent arterioles. ANF dilates afferent arterioles while efferent vascular resistance is increased[64,65] or unchanged.[66] This elevates glomerular hydrostatic pressure and GFR without increasing glomerular plasma flow.[4] The increase in GFR elevates flow rate in the renal tubules and tends to "flush" salt and water down the nephron.

Changes in GFR alone do not entirely account for the diuretic action of ANF. Recent studies[67] indicate that ANF also increases hydraulic pressure in the medullary descending and ascending vasa recta. The elevated pressure in the vasa recta would retard the absorption of sodium from medullary collecting ducts and would favor

the net addition of salt and water to the collecting duct from the papillary interstitium.[4] This process combined with enhanced glomerular filtration may mediate the renal effects of ANF. Interestingly, the diuretic actions of ANF are antagonized by dopamine receptor antagonists, particularly D_1 receptor antagonists.[69]

The atrial peptides elicit diuretic responses in many animal models. Acute administration of ANF increases the urinary excretion of salt and water in normotensive; one-kidney, one-clip; two-kidney, one-clip; deoxycorticosterone-salt and spontaneously hypertensive rats.[51,70] Chronic infusion of ANF for seven days via osmotic minipumps also produces a diuresis in one-kidney one-clip hypertensive rats.[71] However chronic infusion of ANF in normotensive rats as well as in two-kidney, one-clip and spontaneously hypertensive rats fails to increase urine volume, even though systolic pressure is significantly reduced in the hypertensive rats.[72,73] In a model of low-output heart failure in anesthetized dogs, intrarenal infusion of ANF produces similar increases in GFR, as in normal anesthetized dogs,[61] but the diuretic and natriuretic responses to the peptide are attenuated in the low-output dogs. Altered renal responses also occur in a chronic model of low-output heart failure, the vena caval constricted dog.[53] Intravenous infusion of ANF elevates urine volume and potassium excretion, but sodium excretion is unaltered.

Hemodynamic effects - Although the natriuretic actions of ANF may contribute to chronic hypotensive properties, unlike most other diuretics, acute reductions in arterial pressure are regularly observed after administration of the atrial peptides. The hypotensive responses to ANF are also observed in bilaterally nephrectomized rats, indicating that the fall in arterial pressure is independent of the diuretic actions of the peptide.[74] Instead, these depressor responses are due to direct effects on the cardiovascular system.

The depressor action of ANF is observed in rats, dogs and man. Modest reductions in arterial pressure are generally noted in conscious animals, while the hypotensive responses to ANF are markedly potentiated in anesthetized animals.[75] In addition, the hypotensive effects of ANF are enhanced in several models of hypertension. Acute or chronic administration of ANF in normotensive rats causes little or no change in arterial pressure.[51,71-73] However, marked reductions in arterial pressure are observed after ANF administration in one-kidney, one-clip; deoxycorticosterone-salt; two-kidney, one-clip and spontaneously hypertensive rats.[51,70-73] Of the hypertension models tested, deoxycorticosterone-salt rats are the most responsive to the depressor actions of ANF. Finally, as with the diuretic actions of the peptide, bolus injection of ANF elicits transient hypotensive responses, while continuous infusion of the atrial peptides produces sustained reductions in arterial pressure.

Synthetic ANF is a potent relaxant of precontracted isolated vascular smooth muscle from rabbit and rat. The peptide produces concentration-dependent relaxation in both agonist (norepinephrine, angiotensin II, histamine, serotonin)- and KCl-contracted aortic smooth muscle.[9,18,76-78] However, ANF is a more potent vascular relaxant against agonist-induced contractions (IC_{50} values ca. 1 nM) than KCl-induced contractions (IC_{50} values ca. 25-80 nM).[9,78] The *in vitro* relaxant effects of ANF are dependent on the source of vascular tissue. For example, rat[76] and rabbit[77,78] aorta are very responsive to ANF

compared to smooth muscle from other large arteries and veins. The vascular specificity of ANF is also observed in vitro in smaller resistance vessels. ANF relaxes precontracted arcuate arteries from the kidney, but has no effect on arteries of similar lumen size obtained from the mesenteric, coronary, cerebral or femoral vascular beds.[79] These studies suggest that the atrial peptides are not general vasodilators. Indeed, it would appear that ANF affects only certain vascular beds, such as the kidney.

The regional vasodilator actions of ANF are also observed in vivo. Though there are exceptions,[80] bolus injection of ANF generally increases renal blood flow in dogs[81] and rats[7,82,83] without altering blood flow in the coronary, mesenteric, femoral, cerebral and other vascular beds. The renal vasodilator responses are dose-dependent, occur immediately after injection of the atrial peptides and persist for a short time.[83] Interestingly, continuous infusion of ANF frequently fails to elicit sustained renal vasodilator responses. In conscious and anesthetized dogs[52-55,61] and rats[83-85] intrarenal or intravenous infusion of ANF may cause a transient increase in renal blood flow. However, during infusion steady-state conditions, renal blood flow returns to or below control levels in most, but not all[86] studies. In isolated perfused kidneys devoid of exogenous vasoconstrictor tone, addition of ANF to the renal perfusate increases renal vascular resistance.[18]

Interestingly, the hypotensive actions of ANF are often associated with a fall in cardiac output and increased or unaltered total peripheral resistance.[84,88-91] These effects are observed in rats and dogs during continuous infusion of the peptide and are particularly obvious in conscious animals. It appears that dose-dependent reductions in cardiac filling pressure rather than negative inotropic effects accounts for the reduction of cardiac output.[84] Thus, in several animal models, alterations in cardiac output, not vasodilation, are responsible for the acute depressor effects of ANF.

ANF effectively antagonizes the vasoconstrictor effects of norepinephrine, angiotensin II and serotonin in vitro.[9] In addition, pressor responses to norepinephrine, and angiotensin II are attenuated in pithed rats[92] and conscious rats.[93] The depressor actions of ANF are also enhanced in angiotensin II-dependent (high renin) hypertensive rats.[51,94]

Endocrine effects - In addition to direct effects on the kidney and vascular smooth muscle, ANF also exerts profound effects on several endocrine systems. Crude atrial extracts[96] and synthetic ANF reduce adrenocorticotropic hormone (ACTH) and angiotensin II (AII)-induced aldosterone secretion in isolated rat[97-100] and bovine adrenal cells.[101] ANF also inhibits K^+-stimulated aldosterone production in rat adrenal cells[98,99] and in prostaglandin and forskolin treated bovine adrenal glomerulosa cells.[101] A reduction in aldosterone production is noted after synthetic ANF in conscious normotensive rats infused with AII[100] and in high renin hypertensive rats.[102] ANF is reported to reduce glucocorticoid secretion in bovine[100] but not in rat fasciculata cells.[97,98] Investigations into the site of ANF inhibition of AII-induced steriodogenesis in rat adrenal cells indicate an effect on both the early[98,103] and late[98] pathways. An increase in testosterone production from mouse interstitial cells is also observed with synthetic ANF.[104]

Compensatory increases in the release of renin from the kidney are observed with conventional diuretic therapy. The atrial peptides are unique in that in several experiments, infusion of ANF decreased the renin secretory rate.[54,55] The effects of ANF on renin release are commonly observed when renin secretion is elevated, as in the case of pentobarbital anesthetized dogs, but may be undetectable in animals with low or normal renin levels.[52] Inhibition of renin release by ANF is observed in vitro in kidney slices, suggesting that the effects are due to direct effects of the peptide at the macula densa.[95]

Synthetic ANF induces the release of arginine vasopressin (AVP) from the isolated posterior lobe of rat hypophysis in vitro, presumably via specific ANF receptors.[105] Conversely, intravenous infusion of ANF inhibits dehydration and hemorrhagic-induced release of AVP in rats.[106] Dehydration experiments in rats also reveal a decrease in ANF-like immunoreactivity within select brain regions associated with the hypothalamo-neurohypophyseal tract.[107]

Summary - Atrial natriuretic factor represents a new and novel mechanism for controlling arterial pressure and extracellular fluid volumes. Even though the physiological role of ANF remains to be determined, the pharmacology of the peptide indicates that it eventually may lead to the discovery of effective agents for the treatment of edema in patients with congestive heart failure, hypertension, renal insufficiency or hepatic cirrhosis. Unlike current diuretic therapy, ANF may prevent compensatory increases in renin release and may be a potassium-sparing diuretic, through direct antagonism of aldosterone release. ANF may also possess advantages over many vasodilator agents, in that reflex tachycardia and urinary salt retention are not generally associated with the depressor actions of the atrial peptides. Indeed, initial clinical trials with ANF indicate that the atrial peptides are quite effective in humans.[108-109] However, additional clinical trials are needed before the therapeutic potential of ANF can be fully assessed.

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Section VI — Topics in Chemistry and Drug Design

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Chapter 27. Bioisosterism in Drug Design

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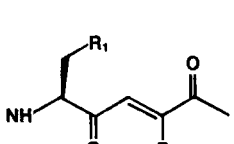
Introduction — This chapter reviews progress in the use of bioisosterism in drug design since the review by Thornber in 1979.[1] Early comprehensive reviews are those by Hansch,[2] Friedman[3] and Burger.[4] The broadest definition of bioisosteres as "groups or molecules which have chemical and physical similarities producing broadly similar biological properties" is used.[1] A major trend in this area is the increasing prevalence of "non-classical isosteres" moieties which do not have the same number of atoms, but which produce a similarity in a key parameter. As pointed out by Thornber and illustrated in this review, similar effects in two functional groups does not imply atom upon atom overlap.[1]

Bioisosterism is part of the spectrum of QSAR as discussed by Hansch[5] and Martin.[6] The concept may be most useful to the medicinal chemist at the early lead seeking stage when sufficient data are lacking to permit a quantitative analysis, or towards the conclusion of synthesis in a series when a transition to a "new" series is desired.[6] In the latter case, other alternative strategies toward development of a new series include QSAR analysis of outliers[6] and distance geometry QSAR.[7] In theory, bioisosterism lends itself to computer substructure searching especially as a means of developing new leads or new series.[1] In practice, examples of new lead generation using bioisosteric principles are few.[8] Papers clearly delineating biosteric relationships in sufficient quantity could make computerized retrieval of bioisosteres a reality. Quantitation of the physical organic relationship between "non-classical isosteres" is enhanced by the growing x-ray literature on binding of functional groups to macromolecular moieties (Chapter 28). In the following sections, examples of bioisosteric relationships are grouped by functional group class.

Peptide and Dipeptide Bioisosteres — Isosteric replacements for the peptide bond have been summarized.[9,10] Pepstatin-related dipeptide analogues have been reviewed.[11] Peptide isosteres have been discussed in reviews on the rational design of peptide hormones and neurotransmitters[12] and conformational restrictions of biologically active peptides.[13] Linear modified retro-peptides have been reviewed.[14] Ketovinyl and hydroxyethylidene dipeptide isosteres (**1**, **2**)[15,16] as well as heterocyclic (**3**)[17] and unnatural amino acid (**4**)[18] surrogates for a peptide β-turn have been reported.



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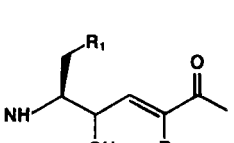

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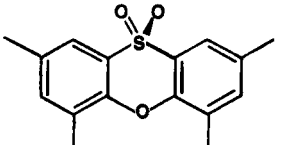

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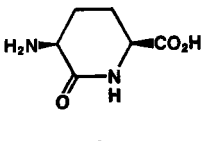

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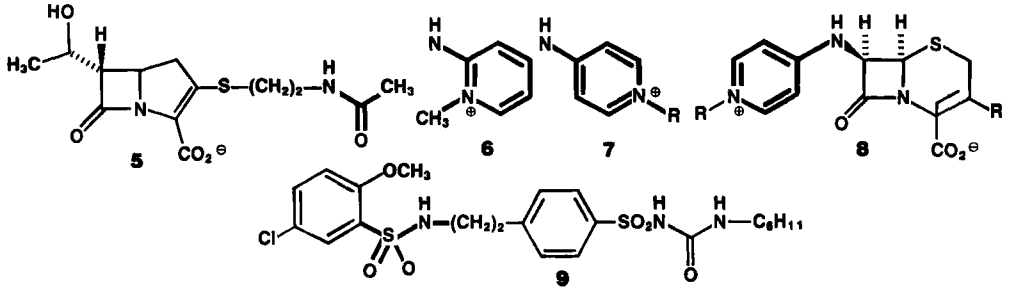
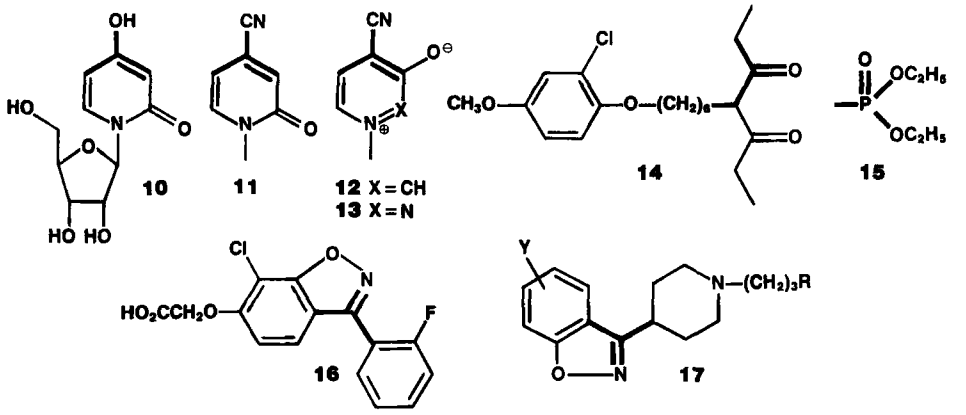
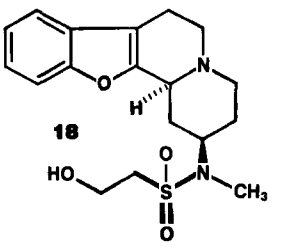
Amide Carbonyl Group Bioisosteres — The N-acetylcysteamine side chain of thienamycin (**5**) was replaced by 2- and 4-substituted quaternary pyridines (**6**, **7**). Similarly, the classical acylamino side chain in cephems was replaced by a 4-substituted quaternary pyridine to give **8** having broad spectrum antibacterial properties.[19] This substitution can be viewed as an extension of the arenology principle in which heteroaromatic nuclei (arenes) are viewed as analogous (arenologous) to classic functional groups.[20] The bioisosterism between benzamide and benzenesulfonamide groups led to the synthesis of hypoglycemic sulfonyl isosteres (**9**) of glybenclamide.[21].



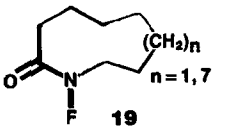
Ketone Carbonyl Bioisosteres — Replacement of the carbonyl-like imide oxygen of 3-deazauridine (**10**) with cyano in analogs **11**, **12** did not lead to antibacterial activity.[22] However, antibacterial activity was detected in mesoionic pyridazine (**13**).[23] The isosteric similarity between ketone and phosphonate groups led to the preparation of the active phosphonate analog (**15**) of the antiherpetic arildone (**14**).[24] The bioisosteric relationship between benzoyl and the 1,2-benzisoxazole moiety has been employed in the synthesis of diuretics (**16**) related to (4-acylphenoxy)acetic acids.[25] A similar relationship can be discerned in neuroleptic (**17**)[26] and in benzisoxazole analogs of haloperidol and benperidol.[27,28]



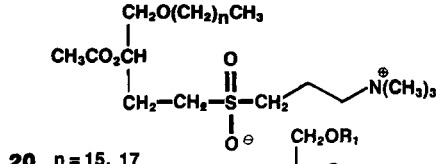
Ester Carbonyl Bioisosteres — The hypothesis that a sulfonamide acts as a rauwolscine ester surrogate formed the rationale for design of the enantioselective α_2 adrenoreceptor antagonist **18**.[29]



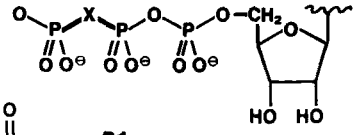
Ester Ether Oxygen Bioisosteres — The premise that the fluorine atom in an N-fluoroamide might substitute stereochemically for one of the non-bonded electron pairs on the ether oxygen of an ester led to N-fluoroazocycloalkanones (**19**) having lactone-like physical and organoleptic properties.[30]



Phosphate Bioisosteres — The general approach to isosteric phosphonates in which the C-O phosphate monoester bond is replaced by the stable C-C bond,[31] as well as approaches to nucleotide[32] and platelet activating factor (PAF)[33] analogues have been reviewed. Replacing the phosphate moiety in PAF with a sulfonyl bismethylene group (**20**) led to loss of platelet aggregating and substantially reduced hypotensive activity.[34] Intact nucleotides cannot enter cells and are readily cleaved extracellularly to the nucleoside. Attempts to find phosphate group replacements have been largely unsuccessful.[35] Isosteric ATP analogues (**21**) were prepared and exhibited an increasing correspondence in physical properties to those of ATP in the sequence X = CH_2<CHF=CCl_2<CF_2=NH<O.[36] Size factors are more important in determining RNA polymerase substrate activity, while isopolarity is more important for hexokinase inhibitory activity. Additional isoelectronic but not isosteric ATP analogues (**22**) exhibit ^{31}P NMR spectra and ionization behavior suggesting that they are good isopolar analogues of ATP.[36] Peroxy analogues such as **23** may be useful in studies of nucleotide requiring enzyme systems.[37] Replacement of the diphosphate bridge in UDP-Glucose with an isosteric $OCONHSO_2O$-residue led to **24** which exhibited antiviral activity in an HSV-1 plaque assay and inhibited glycosylation of proteins in HSV-1 infected Hela cells.[38]



20 n = 15, 17



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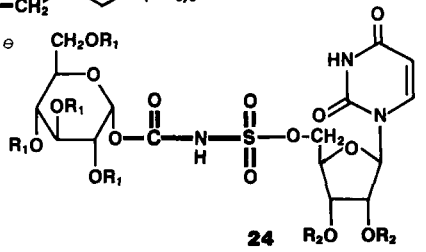
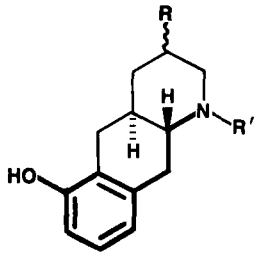
22 X = C≡C, C=C
23 X = O—O

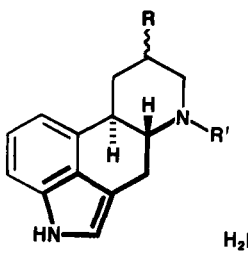


24

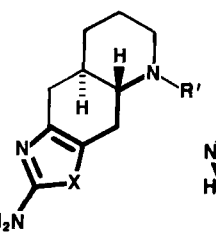
Catechol Bioisosteres — The phenolic ring in dopamine agonists, apomorphine (APO) and (±) CV 205-503 (**25**)[39] has been replaced by heterocyclic moieties. By comparing absolute configurations, the rigid pyrrole ethylamine portion in pergolide **26** was shown to correspond to the rigid dopamine in APO.[40] Similarly the absolute configuration of the active (–) enantiomer **27** (CQ 206-962) corresponds to that of ergoline CQ 32-084 (**28**).[41] Potent stereoselective dopamine D-2 agonist activity in the related heterocycles **29**, **30** and **31** supports the bioisosterism of the aminopyrimidine, aminothiazole and pyrazole rings with the phenol of **25**.[42,43] The hypothesis that the pergolide indole moiety might be a bioisostere for the "meta" OH of dopamine, led to preparation of dopamine agonist **32**.[44] Similarly, replacing the meta phenol moiety in dopamine agonist 3-PPP led to the 4-substituted indole dopamine agonist, RU 2751 (**33**).[45] Replacing the dopamine meta hydroxyl by an indolone system as in **34** led to potent D_2 receptor agonist activity.[46] However, among related carbostyril derivatives (**35**), D-2 receptor potency was not enhanced as with corresponding indolones, and D-1 receptor agonist activity was reduced or lost.[47]



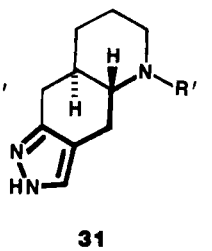
25 R = —CH_2SCH_3
27 R = ⁞⁞⁞ $NHSO_2N(Et)_2$



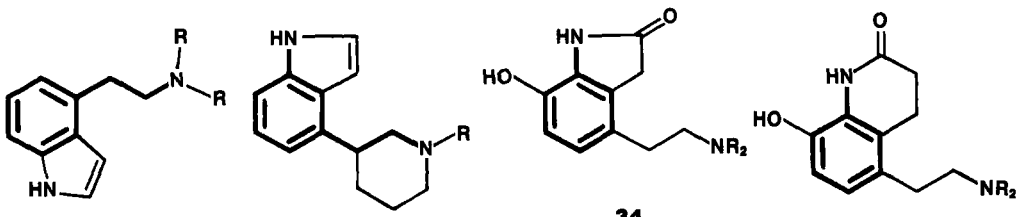
26 R = —CH_2SCH_3
28 R = ⁞⁞⁞ $NHSO_2N(Et)_2$



29 X = N=CH
30 X = S

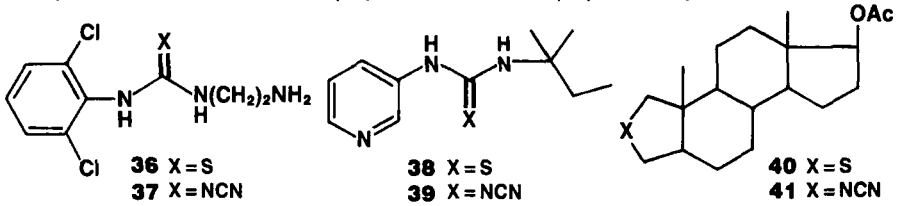


31



32 33 34 35

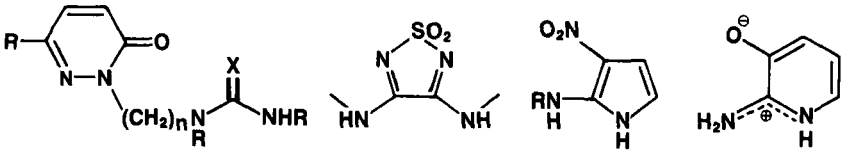
Urea, Thiourea Bioisosteres — Among antihypertensive thioureas (**36**), replacement by cyanoguanidine (**37**) resulted in loss of activity;[48] however, in another series (**38, 39**) hypotensive activity was increased 250-fold by this replacement.[49] Thiourea, cyanoguanidine equivalence led to a successful replacement of a sulfide -S- by -N(CN)- in androgenic 2-heteroandrostanols (**40, 41**).[50] Among antisecretory pyridazinones (**42, 43**) structural requirements for gastric antisecretory activity were very similar.[51] The 3,4-diamino thiadiazole dioxide molety (**44**) was identified as a weakly acidic urea equivalent.[52] Cyanoguanidine or the similar 1,1-diamino-2-nitroethylene groups were replaced by cyano amidine and carbamoyl amidines.[53] SAR additivity differences were noted when related bioisosteres were applied in several H_2-antagonist series,[53] and SAR patterns were rationalized in terms of dipole moment orientation of related bioisosteric groups.[54] Dipole moments[55] and dielectric properties[56] of polar compounds having charge separated zwitterionic species due to resonance (**45**) or tautomerism (**46**) were reported.



36 X = S
37 X = NCN

38 X = S
39 X = NCN

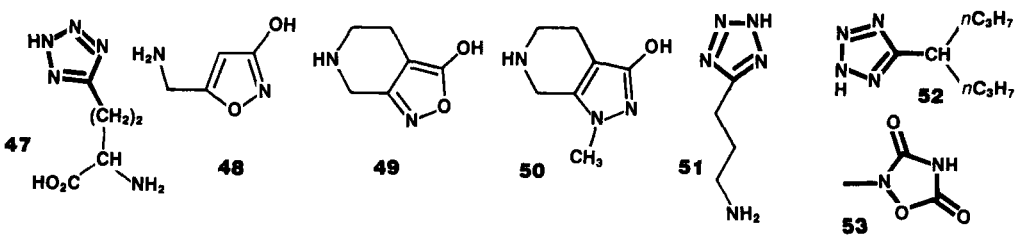
40 X = S
41 X = NCN



42 X = S
43 X = NCN

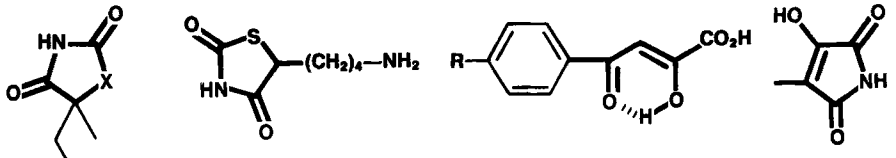
44 45 46

Carboxylic Acid Bioisosteres — The medicinal chemistry of tetrazoles has been reviewed.[57] Abstraction of hydrogen α to a carboxyl group without activation as an AMP ester or coenzyme A thioester is chemically unfavorable. The inability of the tetrazolyl group to undergo a similar activation led to preparation of pentapeptide inhibitors of vitamin K-dependent carboxylation containing the glutamic acid tetrazole analog **47**.[58] The design of GABA agonists, antagonists and uptake inhibitors which contain acid surrogates has been reviewed.[59] Heterocyclic phenols related to the GABA agonist muscimol (**48**) and encompassing an acidity range from 3.0 (ISOTHIP **49**) to 7.1 (**50**) were tested for inhibition of GABA receptor binding.[60] The GABA tetrazole analog (**51**) inhibited GABA-transaminase, but not succinic semialdehyde dehydrogenase (SSA-DH). A difference in the local charge density between the two enzymes in the area of interaction with the tetrazole was proposed.[61] The tetrazole (**52**) and 3,5-dioxo-1,2,4-oxadiazolidine (**53**) analogs of valproic acid were inhibitors of SSA-DH, suggesting that the latter group was also a carboxylic acid bioisostere.[62]

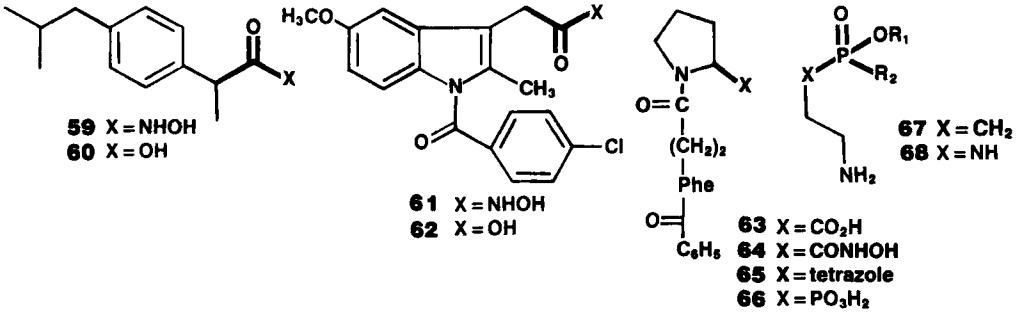


47 48 49 50 51 52 53

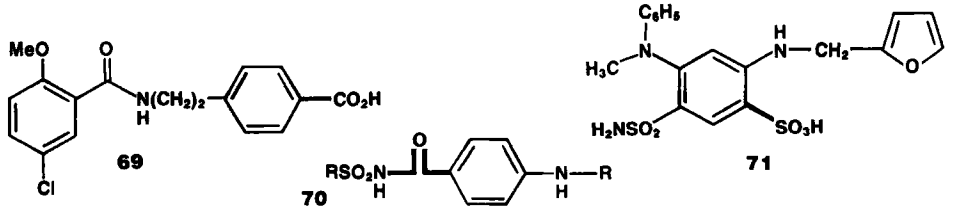
The hypothesis that weakly acidic imide heterocyclic rings found in antiepileptic drugs could function as carboxyl equivalents, combined with the pentobarbital-like effects of the GABA-like cyclic imide quisqualamine on spinal neurons, led to convulsant GABA succinimide and hydantoin analogs (**54** and **55**).[63] Thiazolidinedione (**56**), prepared as an analog of epsilon aminocaproic acid, exhibited antifibrinolytic activity and slightly inhibited activation of plasminogen.[64] A comparison of the aromatic ring glycolic acid oxidase inhibitory SAR between 3-hydroxy pyrrole-2,5-dione **58** and acid **57** suggested a similar interaction with an enzyme site, even though the two adjacent acid functions were not superimposable.[65]



A number of NSAI hydroxamic acid analogs, including ibuproxam-ibuprofen (**59**, **60**), oxamethacin-indomethacin (**61**, **62**) exhibit anti-inflammatory activity in man. While **59** is metabolized to **60** in man,[66] **61** is metabolically stable in man, and is a true bioisostere rather than prodrug.[67,68] Among carboxylic acid replacement analogs of the ACE inhibitor (**63**), best activity was found in hydroxamic acid (**64**). Corresponding tetrazole (**65**) and phosphonic acid (**66**) analogs were less potent.[69] Weak $GABA_B$ affinity and anticonvulsant effects were found in GABA phosphorous analogs **67** and **68**.[70]



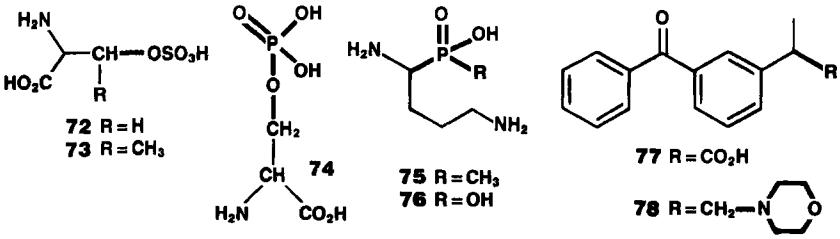
The SO_2NHCO moiety was proposed as a carboxyl isostere based on similar hypoglycemic activities of gliburide and carboxylic acid (**69**).[71] Among analogues of the antiatherosclerotic carboxylic acid cetaben, sterol and triglyceride lowering activity was found in acyl sulfonamides of the general structure (**70**).[72] Replacement of the carboxylic acid in furosemide with a sulfonic acid led, after SAR optimization, to derivative (**71**) which retained oral diuretic activity in dogs.[73]



The "essential" aspartic acid residue in the C-terminal tetrapeptide of gastrin was replaced by the electronically equivalent serine O-sulfate ester (**72**).[74] Among cholecystokinin heptapeptide analogues, unexpected activity increases were observed with analogues containing **72** as well as threonine O-sulfate (**73**) and hydroxyproline O-sulfate.[75] Serine O-phosphate (**74**) as a glutamic acid replacement in the peptide Boc-O-phospho-Ser-O-phospho-Ser-Leu-Ome led to an inhibitor of the vitamin K-dependent carboxylation of Phe-Leu-Glu-Glu-Leu.[76] Replacement of the carboxyl group with a stable phosphinic or phosphonic acid group is exemplified by analogues (**75**, **76**) of ornithine which weakly inhibited rat liver ornithine decarboxylase.[77] Boronic amino acid analogs or their difluoroborane precursors were prepared as chymotrypsin and elastase inhibitors based on the isoelectronicity of the boronic acid moiety with a protonated

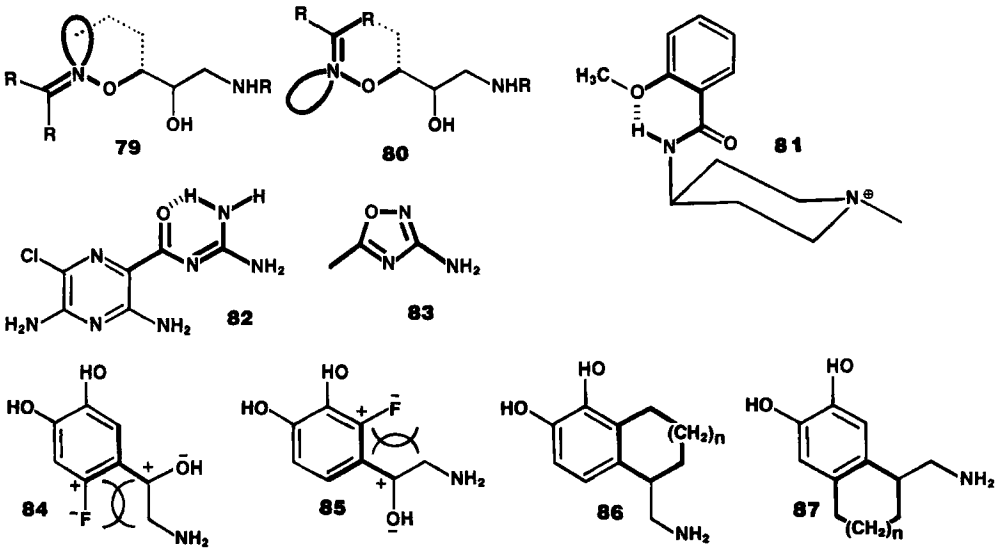
carboxyl group. Phenyl alanine, phenyl glycine, alanine, valine and isoleucine derivatives were active.[78]

Among examples of non-acidic acid isosteres are a neutral terminal hydroxymethylketo group which replaced a carboxylate group in a series of gastric acid antisecretory PGE_2 analogs.[79] Replacement of the carboxyl group in ketoprofen (**77**) with bulky amines as in (**78**) was reported to lead to anti-inflammatory activity.[80] A striking similarity between cimetidine which contains only basic and neutral moieties and GABA on binding parameters at the GABA receptor complex has been reported.[81]

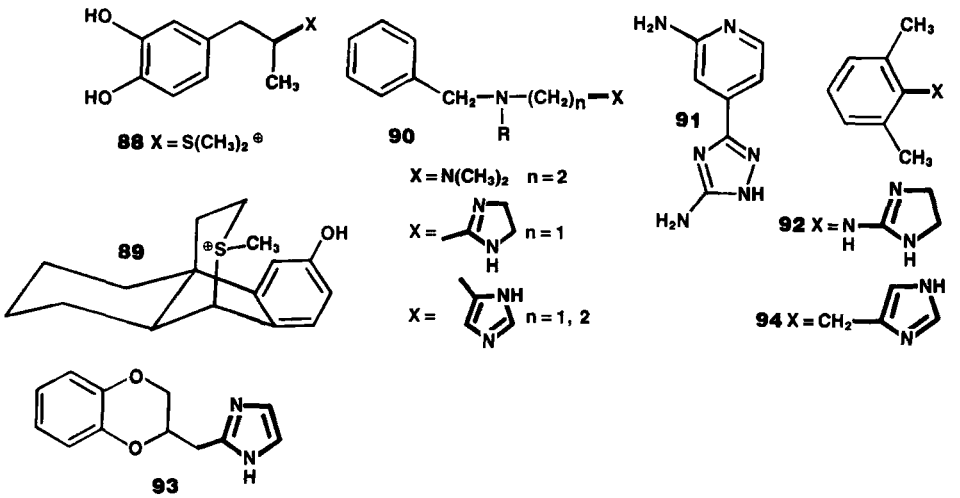


Ring Equivalents — Preferred phenyl ring π electron orientations in x-ray structures have been described.[82] The hypothesis that appropriately positioned heteroatom π electrons could function as aromatic equivalents was combined with x-ray results suggesting that the aryloxymethylene portion of a β-blocker side chain could mimic a portion of an aromatic ring. This led to the proposal that the $C=NOCH_2$ group among stable oxime β-blockers could simulate an aryl group or aryloxymethylene group in either of two possible planar conformations (**79**, **80**).[83] Reduction of the imino bond results in a decrease but not loss of activity and ether derivatives retain activity.[84].

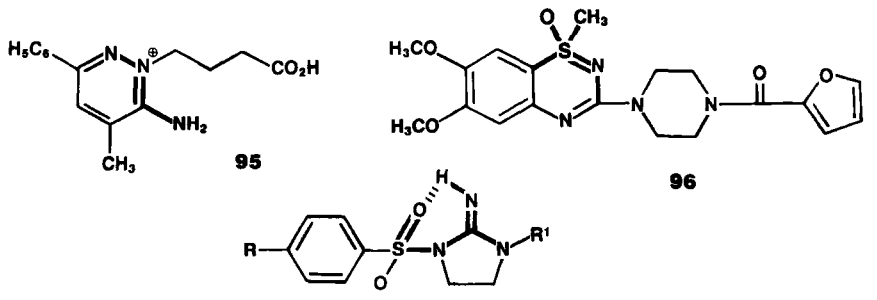
The six-membered H-bonded ring between the imide N-H and *ortho* methoxy oxygen in the benzamide neuroleptic (**81**) was proposed to bind to a dopamine receptor site normally occupied by a phenyl moiety.[85] The likely hydrogen bond between the carbonyl oxygen and guanidine hydrogen in the diuretic amiloride (**82**) coupled with the known conformational integrity of acylguanidines led to the preparation of (**83**) which retained very similar diuretic properties.[86] The hypothesis that electrostatic repulsion between an aromatic fluorine and a side chain hydroxyl group could induce a conformational preference in norepinephrine analogs (**84**, **85**) led to synthesis of carbocyclic derivatives (**86**, **87**).[87]



Basic Groups — Bioisosteric substitution of the ammonium group by a sulfonium group finds application in studies on dopamine agonists such as **88**[88] and the isolevorphanol opiate analog **89**.[89] The bioisosteric relationship between aminoalkyl, 2-imidazolidine and 4-imidazole moieties in histamine H_1 receptor antagonists (**90**) and the similarity between amidines and 2-amino pyridines led to bioisosteric design of the histamine H_2-receptor antagonist (**91**).[8] A similar parallel between such moieties can be discerned in hypotensive agents related to piperoxan and the clonidine analog ST 95 (**92**). Thus, imidazole **93** was identified as a selective α_2-adrenergic antagonist,[90] and MPV-207 (**94**) was a potent hypotensive agent in rats.[91]



Incorporating a GABA amine group into an aminopyridazine gave $GABA_A$ antagonist, SR 95103 (**95**).[92] The recognition that a basic sulfoximine moiety could replace the amidine moiety in prazosin led to synthesis of **96** having equipotent hypotensive activity in animals.[93] Based on the biological profile, structural considerations, and x-ray data, the hydrogen bonded **97** was proposed to mimic biguanides, and the hydrogen bonded imino N-H was suggested as being similar to the obligatory sulfonamide NH in a sulfonyl urea.[94]



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Chapter 28. X-Ray Crystallography of Drug Molecule-Macromolecule Interactions as an Aid to Drug Design

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Introduction - Contributions from X-ray crystallography toward understanding drug-macromolecule interactions come from the full spectrum of crystallographic studies; i.e. from high resolution crystal structures of small molecules, as well as from determinations of crystal structures for macromolecular systems. The former provide highly detailed information about structural properties and about intermolecular interactions, although its relevancy to drug-target interactions may be difficult to assess. Crystal structure determinations for macromolecular systems provide results that are more readily interpretable in terms of drug-target interactions, but often the structures can be determined only to low resolution, limiting the analysis to inference about the nature of intermolecular interactions.

To illustrate the role of X-ray crystallography in helping to understand drug-macromolecule interactions and the use of that understanding for drug design, we have selected a number of examples of studies of small molecules, of drug-DNA interactions, of protein-DNA interactions, and of protein-substrate/inhibitor interactions.

Small Molecule Studies - Automated X-ray diffractometers for data collection, direct methods for structure determination, and ever more efficient computers and sophisticated software have modified the role of small molecule crystallography in the study of biologically important molecules. The focus has changed from trying to interpret the biological relevance of one crystal structure to that of carrying out more systematic studies, especially for families of compounds for which the macromolecular component has not been well characterized. The Cambridge Crystallographic Data Center files greatly facilitate comparison of structural properties of related molecules for which structures have been determined in different laboratories. In addition, the data base can be used to probe intermolecular interactions to reveal trends that would not be recognized easily or substantiated by analysis of a small number of structures. Murray-Rust and Glusker, for example, have studied the directionality of hydrogen bonding to sp^2- and sp^3-hybridized oxygen atoms and discussed its relevance to ligand-macromolecule interactions.[1]

The steroids are the class of compounds most extensively studied by single crystal techniques. Duax and colleagues have reviewed the crystallographic contributions to understanding steroid hormone action,[2] steroid-protein interactions[3] (Figure 1), and the structural basis for chemotherapeutic action of antiestrogens.[4] Kuroda et al. have reported studies of tamoxifen (an antiestrogen) derivatives with good illustration of the use of X-ray crystallography, relatively simple theoretical calculations and empirical binding studies to probe drug receptor interactions.[5]

The analysis of the structural properties of a series of chemically diverse compounds displaying antiepileptic properties has been used to probe the stereochemical basis of their activity.[6] All of the antiepileptics had two hydrophobic regions of structure. When their conformations were compared with these regions maximally superposed, each compound had two electron-donor groups situated in similar orientations and positions with respect to each other and to the hydrophobic groups. Recent crystal structure determinations of β-carboline analogs by Codding and coworkers have led to the hypothesis that the interaction of β-carbolines with the benzodiazepine receptor probably involves a hydrogen bond acceptor of the receptor and π-π interactions for the unsaturated β-carboline derivatives.[7,8] Using crystallographic results and simple force field calculations, a series of Histamine H_1 receptor antagonists has been studied.[9] The antihistamines appear to interact with this receptor in a conformation characterized by a vector from the center of gravity of an unsaturated ring A to the protonated nitrogen atom; if present, a second ring also interacts stereospecifically.

Eckle and Stezowski[10] determined crystal structures for a series of thyrotropin releasing hormone analogs, analyzed the distribution of hydrogen bond interactions by superposition techniques and postulated a possible CNS receptor model (Figure 2). Considerable conformational similarity was observed among the TRH analogs and also between the TRH analogs and the last three residues of Leu^5-enkephalin found in two crystal structures.[11,12] The conformational similarities were subsequently found to extend to the conformations of Tyr-D-Nle-Gly-Phe-NleS.[13]

In an effort to obtain a long acting preparation, computer graphics techniques employing the X-ray conformation of 2-zinc insulin have been used to design hexamer stabilizing agents, small molecules that contribute to the binding energy and fit in the space at the center of the hexamer between the two zinc atoms.[14] A crystalline 4-zinc insulin preparation is used clinically as a slow acting insulin; based on a crystal structure analysis, Smith et al. have attributed the stability of 4-zinc insulin to the observation that it contains a zinc ion and a chloride ion at the bottom of an 8Å tunnel formed by three parallel α-helicies.[15] There is a considerable conformational change on going from 2-zinc to 4-zinc insulin.

Fig. 1. Steroid-Protein-DNA interaction model.[3] Reprinted with permission of Walter de Gruyter & Co.

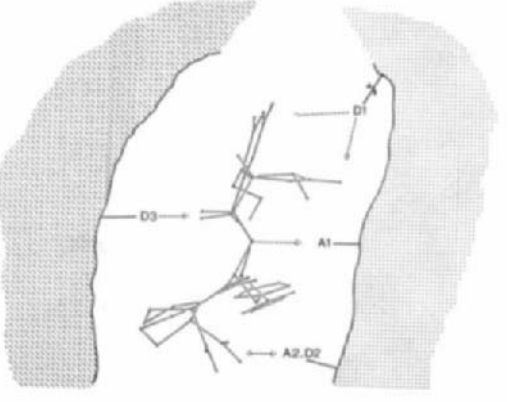

Fig. 2. TRH-CNS receptor model.[10] Reprinted with permission of the American Chemical Society.

Arora has studied the structural properties of rifamycin analogs (DNA-dependent RNA polymerase inhibitors) and has suggested a four-stage model involving metal ion binding, interaction of the chromophore of the rifamycin and aromatic amino acids of the enzyme, interaction between the middle part of the ansa chain and β-sheet of the enzyme, and strong hydrogen bonding interactions between hydroxyl groups of rifamycin and electronegative groups of the enzyme.[16]

In a continuing study of siderophores, van der Helm and coworkers have discussed iron uptake of ferrioxamines in the light of their conformational and topological features.[17]

A highly structured pentagonal water network in crystals of the complex between proflavin and d(CpG) has been described.[18] More recently Teeter reported similar observations in a high resolution structure determination for the hydrophobic protein crambin.[19] A cluster of 16 water molecules in pentagonal arrays was found in a hydrophobic intermolecular cleft in the protein crystal structure. Releasing water of this type may account in part for the entropy contribution to the hydrophobic effect.

Drug-Nucleic Acid Interactions - Berman and Young have reviewed the early literature concerning the interaction of intercalating drugs with nucleic acids.[20]

A number of structure determinations for drug complexes with the oligonucleotide d(CpGpTpApCpG) have been reported, one for daunomycin,[21] a minor groove intercalater, and two for quinoxaline antibiotics,[22,23] which are bis-intercalaters in the major groove. The effects of drug binding on the structure of DNA differ for the two types of complexes. In the daunomycin complex the oligonucleotide maintains Watson-Crick base pairing for all the bases, whereas in the two quinoxaline complexes the AT base pairing is of the Hoogsteen type. There is sequence specific (alanine to guanine) drug-DNA interaction in both quinoxaline complexes. The complex between Triostin A and the octomer d(GCGTACGC) displays the same intercalation site as the hexamer complex, but contains four Watson-Crick and four Hoogsteen base pairs, the latter capping the quinoxaline rings at the ends of the drug molecule.[24]

Crystal structures for complexes of d(CGCGAATT^{Br}CGCG) with netropsin,[25,26] an antitumor drug, and with Hoechst 33258,[27] a potent carcinogen, reveal that both small molecules bind in the minor groove requiring only modest DNA conformational change compared to the uncomplexed oligomer. Dickerson et al. have discussed the use of the structural properties of the complexes for rational design of DNA minor groove-binding anti-tumor drugs.[27] Rubin and Sundaralingam have demonstrated by crystal structure analyses that netropsin and distamycin bind the major groove of the tRNA^{phe} T-stem[28] and that dirhodiumtetraacetate cross links tRNA^{phe}.[29]

A crystal structure determination for cis[Pt$(NH_3)_2${d(pGpG)}] revealed an adduct in which a square planar Pt atom is coordinated to two ammonia N-atoms and two N(7)-atoms of guanosines from the same chain. The results are compared with solution and molecular mechanics studies and the mechanism of action of cis-diaminodichloroplatinum(II) is discussed.[30]

Structural aspects of DNA-actinomycin interactions are still being probed by crystallographic methods. The structure determination for an actinomycin D-d(CpG) complex displayed infinite chains of d(CpG) units connected by Watson-Crick base pairs; actinomycin D molecules lie between the chains.[31] A structure determination for actinomycin cocrystallized

with d(ATGCAT) is in progress, the results of which promise to provide further insight into the structural properties of this system.[32] Sobell has proposed that actinomycin binds to β-DNA.[33,34]

Single crystal structure determinations have provided new insight into the structural flexibility of DNA as shown by the observations of Hoogsteen base pairing, by characterization of Z-DNA[35] and by the fact that mismatched base pairing, even in relativly short oligonucleotides, can be accommodated without large conformational changes in A-DNA.[36]

Model building of drug-DNA interactions is a topic of great current activity. Tautomerism and steric effects of 1-nitro-9-(alkylamino)acridines have been correlated with their antitumor activity and their intercalation geometry in DNA modeled.[37] Models for the structure of anthracycline-DNA complexes,[38-41] and for the interactions of substituted anthraquinones[42] have been proposed based, in part, on crystallographic results. A crystal structure determination for the DNA binding portion of bleomycin A_2 has been used to model its interaction with DNA[43]. Arora has modeled covalent drug-DNA binding for 1,4-benzodiazepine antibiotics.[44] Neidle *et al.* have discussed the structural aspects of sequence specificity of drug-nucleic acid interactions.[45]

Protein-DNA Interactions - Considerable effort is being expended to elucidate the interactions between DNA and proteins by single crystal X-ray techniques. This includes the study of DNA binding proteins,[46-48] modeling the structure of a repressor protein for which no crystal structure is available by considering homology with a known structure,[49] and crystal structure determinations of protein-oligonucleotide complexes.[50,51] Ethylation interference and X-ray crystallographic results have identifled similar interactions between 434 repressor and operator.[52]

A crystal structure determination for a complex of pancreatic RNAse A with $d(pA)_4$ has been reported.[53] At least four oligomers interact with each RNAse molecule in a manner consistent with binding a long single strand of nucleic acid to the enzyme.

Protein-Substrate/Inhibitor Interactions - Thyroid hormones are the only known naturally occurring biologically active iodine containing compounds. The role of the halogens in the thyroid hormones has been examined.[54,55] A model has been proposed based on I···X(N,O,S) intermolecular contacts to explain the unique role of iodine in thyroid hormone-protein interactions.[56] X-ray studies and computer modeling indicate that the pattern of iodine substitution on the thyronine nucleus, the interchange of iodine with similarly sized moieties and the ionization state of the 4'-OH group are all co-operative determinants of protein-hormone binding,[57] and facilitate development of models of hormone conformations at the active site of plasma and nuclear proteins.[58,59] Using prealbumin as a model for the active site of iodothyronine deiodinase, Cody and coworkers[60,61] have shown that the hormone binding site could accommodate many of the potent non-thyroid like inhibitors, *e.g.* the aurone flavonoids (Figure 3). Computer modeling of the thyroxine-prealbumin complex revealed a pocket in the hormone binding site ideally suited to accept a 6'-substituent.[62] The exploitation of these observations provides an attractive route for design of tightly bound hormone antagonists.

In examining the question of the apparent transition-state analog for pepstatin-derived inhibitors of aspartic proteinases, contributions from X-ray crystallography have led to better understanding enzyme-inhibitor complexes.[63] A model for human renin and the renin-angiotensinogen com-

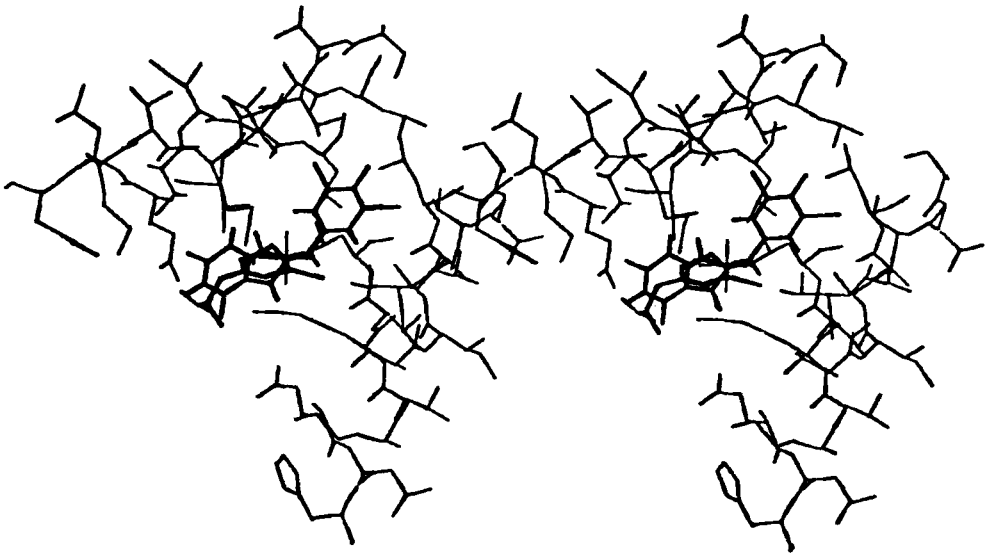

Fig. 3. Stereodiagram of aurone superimposed on T_4 in the binding pocket of prealbumin.[61] Reprinted with permission from Alan R. Liss.

plex has been developed[64] based on sequence homology, structures of aspartic proteinases and that of endothiapepsin inhibitor-H-142 complex.[65] The model indicates that renin may have structural similarities with other aspartic proteinases; certain residues on the periphery of the active site cleft may play a part in the recognition and binding of angiotensinogen.

Dihydrofolate reductase (DHFR) is the primary molecular target of a number of therapeutically important antifolate drugs, methotrexate, trimethoprim (TMP)and pyrimethamine, important in the treatment of cancers, bacterial infections, malaria and other diseases. Crystal structures of lipophilic antifolates have led to an understanding of the stereochemical features required for their antifolate potency and selectivity.[66-68] Natural substrates of DHFR contain a 2-amino-4-oxopteridine moiety,while the most effective inhibitors are 2,4-diamino derivatives of pyrimidine, triazine, pteridine or quinazoline. A comparison of the hydrogen bonding interactions of these compounds in crystals with those in enzyme-inibitor complexes reveals why quinazolines and single ring antifolates could be equally good inhibitors of DHFR.[69] Kraut and Matthews have reviewed the use of X-ray methods as the basis for the design of DHFR inhibitors.[70] Structural results (Figure 4) suggest that analogs containing suitably placed carboxylate groups or a sulfonamide linked to the 3'-position of TMP might interact with Arg-57 in the active site of E. coli DHFR and thereby enhance binding.[71,72] Kuyper et al. designed several such analogs.[73,74] While all were not as effective as TMP as broad spectrum antibacterials, their study demonstrates the enormous potential of this approach to inhibitor design. An iterative receptor based structural approach for designing selective inhibitors has been suggested.[75] Using X-ray results and molecular graphics directly in model building, Ghose and Crippen describe the success of distance geometry methods in developing receptor models for various DHFR inhibitors.[76] Directed mutagenesis experiments on DHFR, in conjunction with accurate crystal structures, help delineate enzyme structure-function relationships and could be of value in inducing useful functional changes in enzyme molecules.[77] Studying DHFR and papain, Hansch, Langridge and coworkers used QSAR and computer graph-

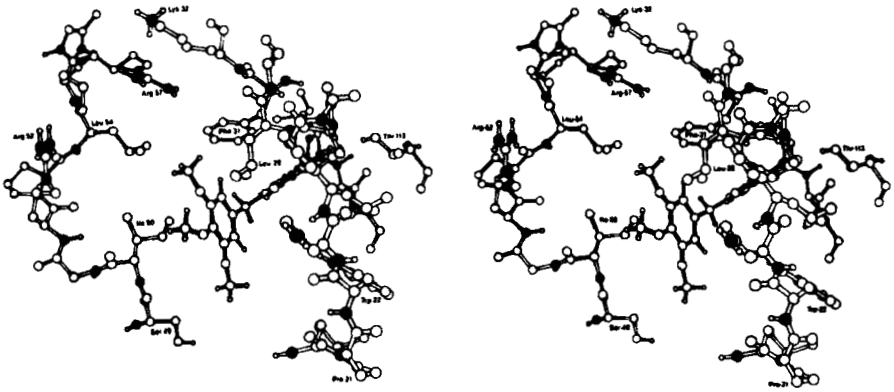

Fig. 4. Stereodiagram of TMP in the binding site of E. coli DHFR.[74] Reprinted with permission of the American Chemical Society.

ics with crystallographically determined receptor structures to provide a detailed molecular model of drug-receptor interaction and generate experimentally verifiable predictions of the activity of new analogs.[78-81]

Purine nucleoside phosphorylase (PNP) is also a target for chemotherapeutic intervention. Bugg and coworkers have identified the PNP active site by crystallographic studies and have correlated the proximity of the major sites for p-chloromercuribenzene sulfonate and ethylmercurithiosalicylate to the substrate binding pocket with their ability to inactivate the enzyme in solution.[82] Phosphorylated nucleosides functioning as multisubstrate analogs are potent inhibitors of PNP; although excluded from cells, these compounds are important vehicles for mapping the active site crystallographically.[83] Correlation of the X-ray and kinetic studies could provide the rationale for design of chemotherapeutic PNP inhibitors.

Thermolysin is a good model for mechanistic studies of physiologically important zinc peptidases. It changes little on inhibitor binding and a variety of crystallographic, substrate, inibitor and chemical modification data are available. Matthews and coworkers have examined the mechanism of peptide hydrolysis by thermolysin with computer graphics and designed inhibitors.[84] The binding of several inhibitors was also studied crystallographically.[85-88] With a good understanding of the common functional requirements of different zinc peptidases, a known inhibitor of one enzyme can be adapted suitably for others.[86]

Rossmann has reviewed structure-function relationships among the NAD-dependent dehydrogenases.[89] Most of their inhibitors bind in the adenine portion of the coenzyme binding site. A comparison of the wide spectrum of NAD, NADP and FAD dependent dehydrogenases shows that a relatively small segment of each protein has a common super-secondary structure.[90] Detailed structural information on the interactions between the enzymes and their coenzymes has helped establish functional and evolutionary relationships. It has been suggested that similarly oriented His-Asp pairs in the active sites of lactate and malate dehyrogenases, serine proteases, thermolysin and phospholipase A_2 may function as proton relay systems.[91]

Goodford[92] and Beddell[93] have reviewed drug design based on receptor fit method. A method has been described in which energy contour surfaces are displayed on a graphics system together with the macromolecular structure, so that energy and shape can be considered together when designing

drugs.[94] Knowledge of the structure of the receptor site allows design of compounds of different chemical type rather than just the modification of an existing enzyme substrate to produce an antagonist. Substituted benzaldehydes, which bind to human hemoglobin at a site different from the natural effector (2,3-diphosphoglycerate), produce an allosteric, non-competitive antagonism.[95] The predicted "best" compound is a potent inhibitor, at low oxygen pressure, of the sickling of erythrocytes and may be clinically useful.

Quiocho and coworkers have studied protein-ligand interactions in periplasmic binding proteins with a view to drug design.[96] Analysis of L-arabinose binding protein (ABP) reveals a picture consistent with the functional requirements of the protein.[97] The liganded form of ABP accommodates both α- and β-anomers of L-arabinose; the structural information provides new understanding of the protein-sugar interaction, a basis for analysis of ABP with altered activity or specificity generated by site specific mutagenesis and for substrate design.[98] The structure of the sugar-binding site of the lac-repressor has been predicted by identification of homologous residues in it and ABP, opening up new avenues of study for this region of the repressor protein.[99]

The digestive enzyme α-amylase is important from a metabolic standpoint and its regulation could assist in control of blood sugar levels and in prevention of tooth decay. McPherson and coworkers have identified the active site of the enzyme and suggested a class of possible inhibitors, the β-thiomaltosides.[100]

Transamination allows the specific study of the function of the α-amino group of proteins by selective modification. The structure of transaminated phospholipase A_2 has resulted in the function of the $\alpha\text{-}NH_3^+$ group being envisaged as locking part of the lipid binding site, by means of a hydrogen bond, into a conformation suitable to bind to phospholipid aggregates.[101] Modifications affecting the hydrogen bond result in part of the lipid binding site becoming mobile or disordered.

The importance of electrostatic interactions, especially involving the macro-dipole of the α-helix, in the design of new drugs based on enzyme crystallography has been discussed by Hol and Wierenga.[102] Amit et al. have reported the first structure of an antigen-antibody complex; it helps visualize the structural basis of cross-reactivities of an antibody with heterologous antigens and the effect of a single aminoacid substitution on antigenic specificity.[103] From the binding of phenylhydrazine to myoglobin, Ringe et al. have delineated one pathway for oxygen or carbon dioxide from the enzyme surface to the iron atom.[104] Blundell and Wood have reviewed the dynamics of polypeptide hormones and highlighted conformational preferences and factors involved in receptor binding.[105] Tulinsky has studied the interaction of several classes of small molecules with α-chymotrypsin.[106]

Huber and coworkers have discussed structural implications for activity of citrate synthetase[107] and for Kazal trypsin inhibitor.[108] Binuclear iron complexes in various forms of the oxygen-transport protein hemerythrin have been studied by Jensen and collaborators.[109,110] Lipscomb and coworkers have studied the binding of a bisubstrate analog to aspartate carbamoyltransferase and suggested a trimer-trimer interface as a possible pathway for homotropic interactions.[111] Phillips has reported studies of triosephosphate isomerase that demonstrate the ability of proteins to change conformation in crystals under the influence of the diffusion of substrate and inhibitors.[112]

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Chapter 29. Recent Developments in Computer-Assisted Organic Synthesis

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Introduction - Computer-assisted organic synthesis planning has, in less than two decades, blossomed from an intriguing curiosity into a practical, useful tool whose purpose is not to replace organic chemists but rather to enhance their creativity and efficiency. This report will concentrate on developments appearing after publication of the previous, excellent reviews,[1,2,39] but will also attempt to present some additional background on the purpose and capabilities of the various major programs.

The term "computer-assisted" has a variety of meanings; "assistance" can range from looking up a specific reaction to transform compound A to compound B, to presenting the chemist with a large number of detailed synthetic routes (including specific reagents and general information on yields for each step) to produce the target from any one of a number of commercially available starting materials. To simplify matters, the programs discussed here are divided into the categories of "passive" and "active", with a final word about some special-purpose programs.

Passive Programs - These programs are basically computerized libraries, providing references to the chemist. Examples are CAS Online,[3] MACCS,[4,5] and MACCS-3D,[4,5] structure-retrieval programs which can locate all compounds in the database that contain a given substructure or even, in MACCS-3D, a given three-dimensional array of atoms which may not necessarily be connected. A number of companies have used the CAS or MACCS systems to set up their own private databases of proprietary compounds.[6]

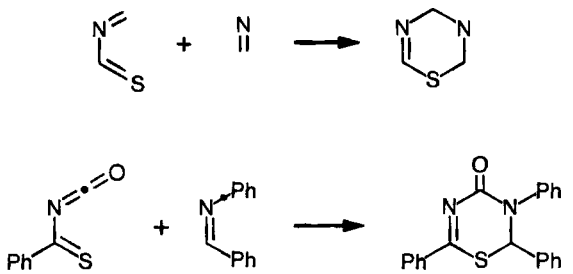
REACCS[4,6] and SYNLIB[7,8,9] are reaction-retrieval programs which will present the chemist with sets of reagents/conditions/references for the execution of a particular transformation. The databases for REACCS are compiled from the "Organic Syntheses" series and from Theilheimer's "Synthetic Methods of Organic Chemistry"; another database, being prepared in collaboration with industry, will contain reactions abstracted from the recent literature.[6] As with MACCS, each user can also encode proprietary reactions in the database. The searches can be keyed to find particular substructures or reaction centers, types of reactions and yields. ChemBase, a program related to MACCS and REACCS that can be executed on a microcomputer, has recently been introduced.[8]

The SYNLIB program, conceived by W. C. Still, has its database drawn from the chemical literature, and is added to regularly by a consortium of academic chemists. A search here is also keyed to a desired substructure in the product, which is entered by the chemist. The "narrowness" of the search will be defined by how specifically or generally this target moiety is represented. The program will then present all reactions which generate the chosen substructure. A future version of the program will allow users to specify features on the reactant side of the entry.[8] SYNLIB was developed for use with personal computers.

Active Programs - These programs perform manipulations (transforms) upon their particular computerized representation of molecules (ensembles, bondsets, unique arrays) either in a retrosynthetic or in a prosynthetic sense. These programs may be interactive, allowing the user to evaluate intermediate results, or may run freely without user interference. Active programs can be divided further into categories described by I. Ugi as logic-oriented or information-oriented.[10]

Logic-oriented - These programs attempt to proceed from first principles in a non-biased, non-empirical manner, by the rearrangement of atoms in an ensemble (molecule) via simple bond-making and bond-breaking processes (connection and disconnection).

Programs developed by Ugi like EROS[11] and IGOR[12] are not intended as a tool to develop new synthetic routes to a given target. Rather, they are an exploration into a general theory of the logical structure of chemistry, via the mathematical recombination of matrices representing molecules or collections of molecules. The human analogy for this process would be the "electron-pushing" drawings which all chemists have used to represent organic reactions. In his own words, Ugi has extended "the concept of isomerism from molecules to ensembles of molecules". While the "chemical reactions" generated by these programs may not represent "realizable chemistry", the program, by exploring all conceivable recombinations, has the capability of generating novel reactions which may inspire the user's creativity. For example, Scheme I shows a "basis reaction" (redistribution of valence electrons) in IGOR and the corresponding literature reaction.[12]



Scheme I.[12]
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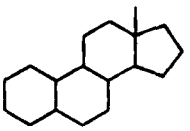
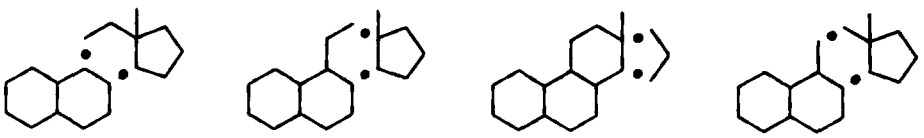
The SYNGEN program, developed and recently reviewed by J. B. Hendrickson, is a retrosynthetic, non-interactive program.[13,40] It generates precursors for a target structure by disconnection of the target skeleton into "bondset families" and correlation of these fragments with skeletons of commercially-available materials. The logic upon which the program is based has been discussed previously.[14] The key to restricting the number of possible routes generated is to require convergency in the forward direction. Only bond-construction reactions are allowed. Multiple constructions (formation of more than one bond in one step) also lend weight to one route over others. In each construction step functionality is generated in precursor skeletons and the target skeleton which will allow an assembly reaction to occur. This is represented by "two linked half-reactions (generated from general mechanistic principles) on each side of the bond formed". Minimization of refunctionalization between construction steps is also sought. Usually, only two levels of "dissection" are necessary. Correlation of a precursor with a compound in the "starting material catalog" halts the program. After a run, the user may impose constraints via a

SYNOUT program to sort extensive output. The program's intent is "to provide a short optimal set of all the shortest, convergent syntheses" of a given target. Input is via a graphics terminal.

The program has been applied successfully to targets such as estrone, jasmone, and ptilocaulin; in these cases it has "found" many of the shortest published syntheses of these molecules.[13] In Scheme II, the "first-level" dissections of an estrone-like skeleton, 1, which were found by the program, are displayed.



1



Scheme II.[13]
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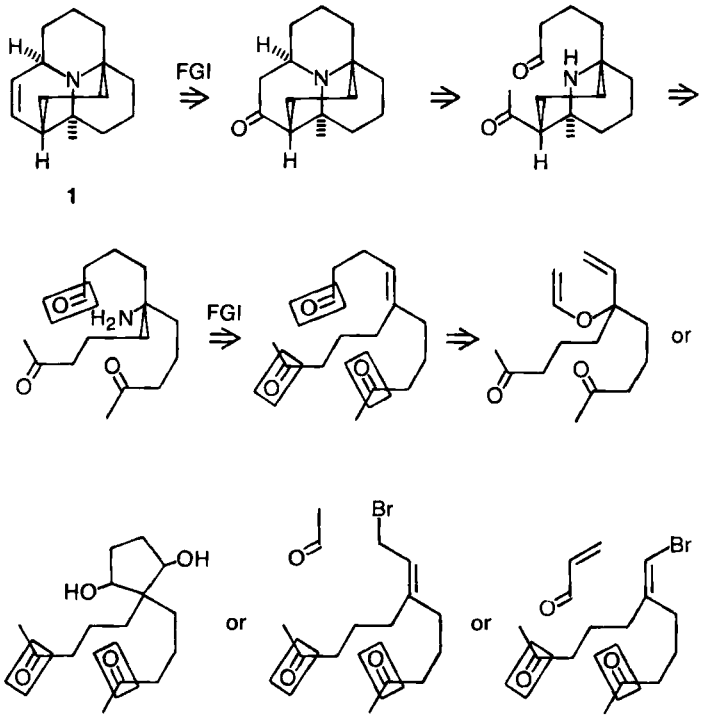
Information-oriented - These programs utilize an internal library (database) of chemical reactions which are usually applied in a retrosynthetic sense, to generate precursors to the selected target. This is an iterative process, with each new precursor becoming, in turn, a target for which further precursors are generated. Thus, an entire "synthesis tree" of precursors and possible synthetic routes is produced. The number of routes generated, and criteria for halting the precursor search, will be a function of the particular program's self-imposed constraints, the chemist's initial choice of strategies (in LHASA) or, in the interactive programs, by intercession of the user.

SYNCHEM, developed by H. L. Gelernter, is a non-interactive program of this type which relies on a database of known reactions (synthetic schemata) and a commercial library as the "possible starting material" table.[15] A match between one of these compounds and a newly-generated precursor halts the search. The program was abandoned in favor of a newer version, SYNCHEM2.[16] This program was developed to deal with stereochemical considerations, and the simultaneous application of a transform at more than one reactive site in the molecule. It may also be used to apply transforms in a pro-synthetic sense. Input is via a Wiswesser-like encoding notation called SLING. The program is under continuing development and has been recently reviewed.[17]

The library-based, interactive retrosynthetic programs (LHASA, SECS, CASP) have proven of great practical value to the organic synthesis chemist in both academics and industry. While not capable of "inventing" new chemistry, the programs can suggest novel strategies in the construction of a particular target (augmented by the chemist's creativity) while providing routes whose individual steps are valid and well-precedented. The user may intercede at any level of precursor-generation to favor or reject certain routes ("prune" the synthesis tree), or to stop the search. Graphical input and output allows the chemist to manipulate structures in a familiar fashion.

OCSS, the original program of this type, was developed at Harvard by E. J. Corey and W. T. Wipke in the 1960's.[18] Evolution in design has led to the current version, LHASA-11 or LHASA, whose capabilities have been reviewed recently by Corey.[19,41] While the basic concept, retrosynthetic analysis of a target molecule via generation of a "tree" of precursors, still remains, some improvements include the addition of more "long-range strategies"; that is, the chemist can call for a target to be analyzed with respect to the application of a particular, powerful constructive reaction, e.g. the Diels-Alder,[20] Robinson annulation,[21] Birch reduction, or halolactonization.[22] These "transforms" can call upon up to 25 chemical reaction "steps" in order to reconcile the target to a precursor obtainable by the selected transform.

Scheme III shows a retrosynthetic scheme generated by LHASA for the alkaloid porantherine (1) based on a functional group strategy. Scheme IV illustrates a portion of the retrosynthetic scheme for valeranone in which the chemist has invoked the Robinson annulation transform. The structure is dissected with a view to generating precursors which can be assembled utilizing this powerful reaction.[19] Note that boxes indicate interfering functional groups which must be protected through the synthetic route. Functional groups can also be interchanged (FGI step) or added (FGA step) by the program in order to carry out its goal.



Scheme III.[19]
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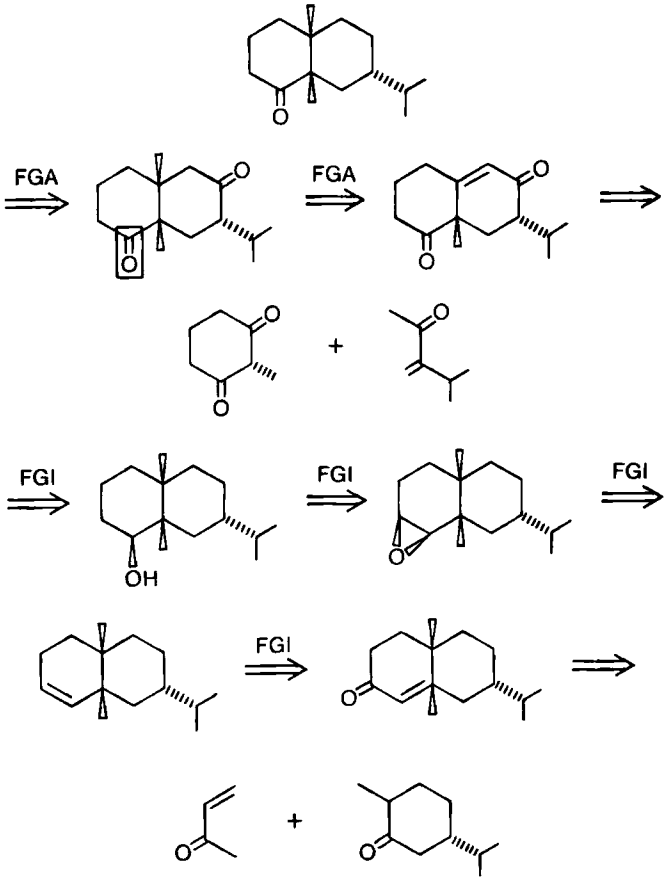

Scheme IV.[19]
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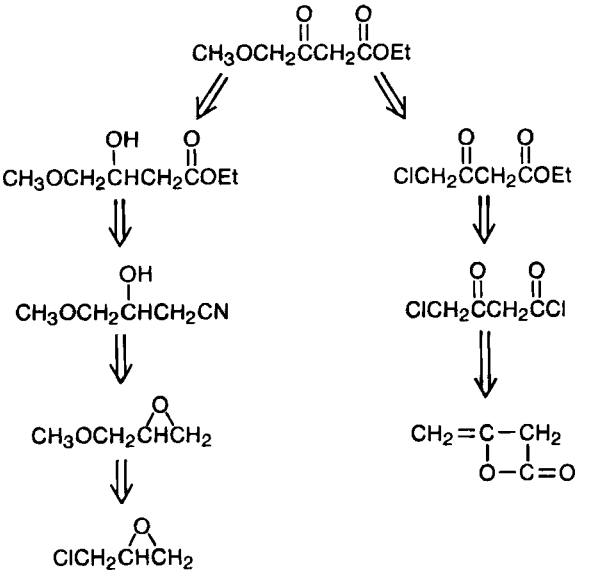
Other significant additions include granting the program the capability to choose the functionality-protecting groups required for the steps carried out in a particular synthetic route.[23] This subprogram, PROTECT, can also stand alone as a protecting-group information-retrieval program. A version of LHASA designed to assist in the teaching of organic synthesis has also been developed.[24]

The SECS program,[25] developed by Wipke, has probably become the program most utilized by the pharmaceutical industry. It is similar to LHASA in its key features: generation of a "tree" of precursors to a given target molecule, and utilization of a "library" of possible synthetic transformations. An important difference is that the chemical transforms are separate from the program itself and formulated in ALCHEM, an English-like computer language created for SECS. Chemists can use this language with minimal instruction to add new transforms to the reaction library. Another program, QED, can be employed to generate goals which then become the controlling strategy for SECS.[5]

The SECS program has become very popular as a practical tool to assist the organic synthesis chemist;[26] an offshoot version (CASP) is being utilized by a consortium of European pharmaceutical companies.[42] Scheme V illustrates the application of the SECS program to the retrosynthetic analysis of a relatively simple molecule. Interestingly, the longer route proved to be of much higher yield.



Scheme V.[5]
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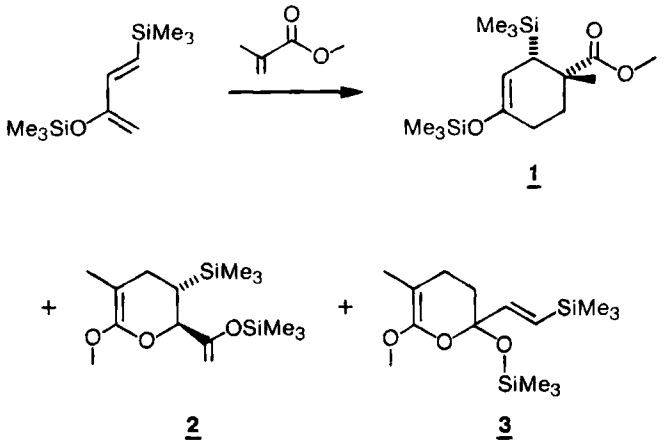
The CAMEO program, developed by W. L. Jorgensen, is different in objective from the previous interactive programs.[27] Given a starting material, reagents and conditions, this program's goal is to predict all possible products of the reaction. This is achieved by first perceiving all the reactive sites in the molecule susceptible to change under the reaction conditions; then applying mechanistic principles to alter the molecule in a prosynthetic sense; and finally, screening the products to rule out (or assign less weight to) impossible (or less-likely) products. Graphical input and output is also utilized here.

The value of CAMEO is three-fold. First, possible side products unanticipated by the chemist planning a reaction may be uncovered, whose generation might be avoided by altering reaction conditions. Second, the basic knowledge acquired by the development of the program's mechanistic guiding principles can provide new insights into the mechanism of organic reactions. Finally, the program is capable of "discovering" new reactions which are mechanistically valid. In the decade since its introduction, CAMEO has undergone considerable development. More subtle considerations of the stereochemical outcome of reactions and of accessibility limits for intramolecular reactions have been incorporated.[28] The program has been designed in a modular fashion, each module concerned with a particular mechanistic category. Initially limited to base-catalyzed and nucleophilic processes, CAMEO today can deal with organometallic chemistry,[29] organosilicon chemistry,[30] pericyclic reactions,[31,32] electrophilic processes, and nucleophilic[33] and electrophilic[34] aromatic substitution.

Scheme VI, from the organosilicon reagent module, illustrates both CAMEO's thoroughness and evaluative ability. While products 2 and 3 are predicted, the computed ΔH's for each are at least 30 kcal/mol less favorable than product 1. Stereochemistry (endo attack) is also accounted for.[30]



Scheme VI.[30]
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Special-purpose Programs - Programs have also been developed which, given a particular target, will simply present a selection of potential starting materials. For example, Wipke has developed SST,[35] an interactive graphical program which selects starting materials by (a) abstracting the target to a carbon-skeleton matrix; (b) abstracting starting materials from a database library; (c) matching closely-comparable abstracts, and (d) presenting the commercially-available starting materials for the chemist's consideration. The user may also impose initial constraints such as requiring the presence of certain features, use of chiral starting materials, or even set limits on maximum prices.

The CHIRON program, developed by S. Hanessian, consists of three subprograms.[43] CAPR dissects a target molecule with respect to its stereochemical features.[36,43] It then attempts to correlate these chiral subunits with readily-available, stereochemically-related chiral precursors via another subprogram, CAPS. The programs will scan the carbon framework, breaking bonds where two non-asymmetric centers are found, to generate subunits of minimum three-carbon length, containing one or more asymmetric center. These subunits are then correlated with database compounds that contain the same number of asymmetic centers and functionality that can be adjusted to obtain the target; thus CHIRON applies the power of a computer to the "discovery" of "chirons" (Hanessian's term for chiral synthons), an old and revered approach to the total synthesis of natural products.[37] This third subprogram draws structures for slides.

Two final programs bear mention because of their potential usefulness to the pharmaceutical industry, even though technically not synthesis programs. XENO,[4,38] a graphical program developed by Wipke, applies known metabolic biotransformations to a starting material (e.g., a drug or food additive) in order to predict possible metabolites of the compound. A "synthesis tree" of metabolites is generated, since each new product is also subjected to the

"reaction conditions". The program includes consideration of the stereochemical selectivity of each "biotransform", and constraints of species selectivity (transformations pertaining to only the desired species) can be applied. METAB will be a program developed to explore specific potential metabolites in a look-up fashion similar to SYNLIB's.[8]

Conclusion - The computer-assisted synthesis programs developed to date seem to be designed for one of two purposes: first, to develop new insights into the basic knowledge of chemistry, from its underlying logical structure to the mechanisms of organic reactions; and second, to provide practical assistance to the organic synthesis chemist, providing potential routes to a target compound, a selection of readily-available starting materials and a means to evaluate reactions with regard to the generation of by-products.These purposes are certainly not mutually exclusive; many programs combine these goals. Future developments in this fast-changing area should reflect the trends of continued reduction in the cost of computer time and hardware, a rise in machine sophistication, power, and "user-friendliness", and a more widespread interest in the application of computers to chemical problems.

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Chapter 30. Tandem Mass Spectrometry for the Identification of Drug Metabolites

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INTRODUCTION

The combination of gas chromatography and mass spectrometry (GC/MS) has become a principal technique for the separation and structural elucidation of drug metabolites.[1-3] Currently, most drug metabolite identification methods are based on the incorporation of radioactive or stable isotopes[4] into the drug molecule, and tracking the tracer by some form of chromatography (thin layer, liquid column, or gas-liquid) in physiological samples from test animals or humans. Selected fractions are then identified by mass spectrometry. Synthesis of labeled drugs is both time-consuming and expensive; thus, a rapid method of metabolite detection that is not dependent on tracer techniques is highly desirable. Tandem mass spectrometry offers such a method.

Tandem mass spectrometry (MS/MS) is a relatively new development in the field of drug metabolite identification; hence, only a few reports of use of this methodology can be found in the literature. In many cases, MS/MS can eliminate the need for chromatography and provide additional capabilities not obtainable with the relatively slow chromatographic techniques in which sample components elute one at a time. Preliminary studies have shown that MS/MS is capable of rapidly (2-3 hrs.) identifying metabolites in physiological samples with minimal sample preparation,[5] and can be used to postulate new metabolite structures without the use of reference standards.[6] This review provides an overview of the application of MS/MS to detection and identification of drug metabolites, and illustrates the use of tandem quadrupole MS/MS (TQMS) with examples from recent research in this field.

TANDEM MASS SPECTROMETRY

A recent book[7] and several recent reviews[8-12] contain extended explanations of the theory, instrumentation, and applications of MS/MS. Described here is the development of MS/MS for mixture analysis, and the unique characteristics and special operating modes of a tandem mass spectrometer which make the system particulary well suited to the rapid detection and identification of drug metabolites.

Mixture Analysis with MS/MS - The potential of MS/MS for the analysis of complex mixtures was first demonstrated in the laboratories of Cooks[12] and of McLafferty.[13] These early MS/MS instruments were reversed-geometry double-focusing mass spectrometers, consisting of a magnetic sector (momentum analyzer) followed by an electric sector (kinetic energy analyzer). The technique was, therefore, referred to as mass-analyzed

ion kinetic energy spectrometry (MIKES). The applicability of tandem quadrupole mass analyzers for mixture analysis and structure elucidation was first demonstrated by Yost and Enke with a triple quadrupole mass spectometer.[14] A conceptual diagram comparing triple quadrupole tandem MS/MS with GC/MS is shown in Figure 1. A tandem mass spectrometer consists of an ionization source, two mass analyzers (Q1 and Q3) separated by a fragmentation region (Q2), and an ion detector. The quadrupole mass analyzers directly provide two stages of mass analysis, since they separate ions on the basis of their mass-to-charge ratio as opposed to momentum-to-charge ratio (magnetic sector) or kinetic-energy-to-charge ratio (electric sector). The center quadrupole, Q2, allows ions of all masses to pass simultaneously. In the presence of an inert collision gas, ions are fragmented by collisionally activiated dissociation (CAD). This configuration provides the high sensitivity, selectivity, and ease of computer control required for the separation and identification of trace compnents in complex mixtures.

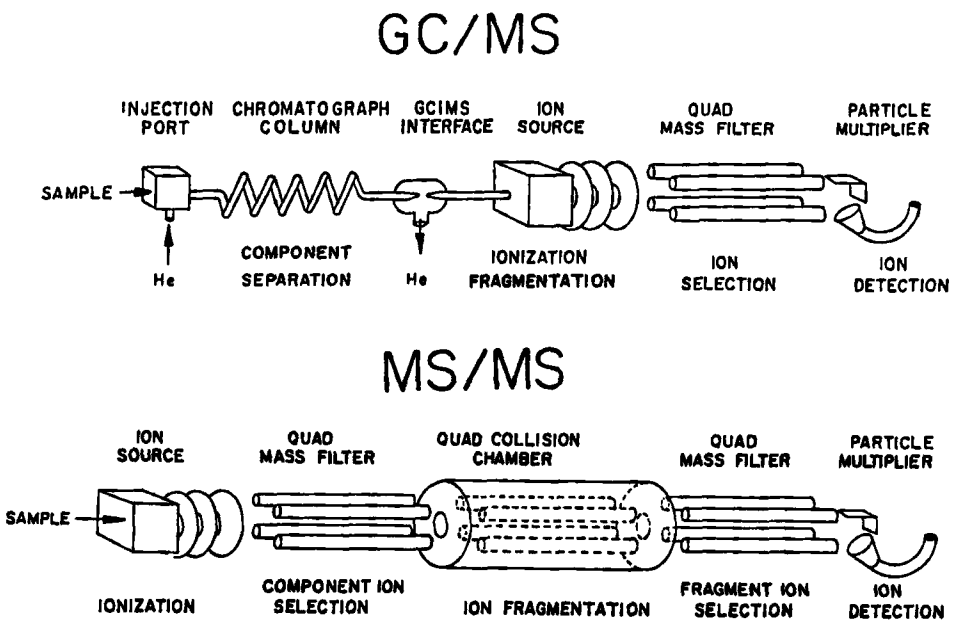

Figure 1. Conceptual diagram of a tandem quadrupole mass spectrometer (MS/MS) and a gas chromatograph/quadrupole mass spectrometer (GC/MS).

Operationally, a sample is introduced into the ionization source of the tandem mass spectrometer, where ions characteristic of the components of the mixture are produced. Separation of the various mixture components is achieved by setting Q1 to pass ions of a particular mass to undergo CAD in Q2. This process yields various daughter ions which are then analyzed by scanning Q3. The resulting daughter spectrum can be used for identification and quantitation of the selected components in the mixture just as can a mass spectrum produced by GC/MS. Since all molecules vaporized from the sample are ionized simultaneously in the ion source, the components of interest can be selected essentially instantaneously for identification or quantitation. MS/MS can thus offer an advantage in speed of analysis over GC/MS, in which the first stage of separation is the chromatographic elution of the components of interest.

Characteristics - There are several characteristics of a tandem quadrupole mass spectrometer which make it suitable for the sensitive, selective, and rapid detection and identification of drug metabolites. High sensitivity results from the high transmission of the quadrupole system, and the very efficient CAD process which occurs in the quadrupole collision chamber, Q2.[14,15] Selectivity in a MS/MS instrument is a function of the mass resolution of the two mass separation stages. Since quadrupoles separate ions by their mass-to-charge ratio (m/z), tandem quadrupole instruments can provide unit mass resolution in both stages of mass separation. Reversed-geometry MS/MS instruments cannot provide unit mass resolution in both stages because of the release of kinetic energy during the CAD process. Each of the scanning quadrupoles, Q1 and Q3, can be operated independently. Under computer-control, the various operational modes of the instrument, as well as other instrument features such as mass range, scanning speed, and collision energy, may be rapidly selected. Another feature of quadrupoles is their ability to scan a mass range very rapidly [0.5 msec/mass unit (u)] or to quickly switch from one mass to another. Despite the wide variety of instruments employed in MS/MS studies[9], all applications to drug metabolite identification have been performed on triple quadrupole instruments.

Operational Modes - A variety of operational modes are possible with a tandem mass spectrometer (Figure 2). The specific operational mode chosen for a particular analysis will depend on the information desired.

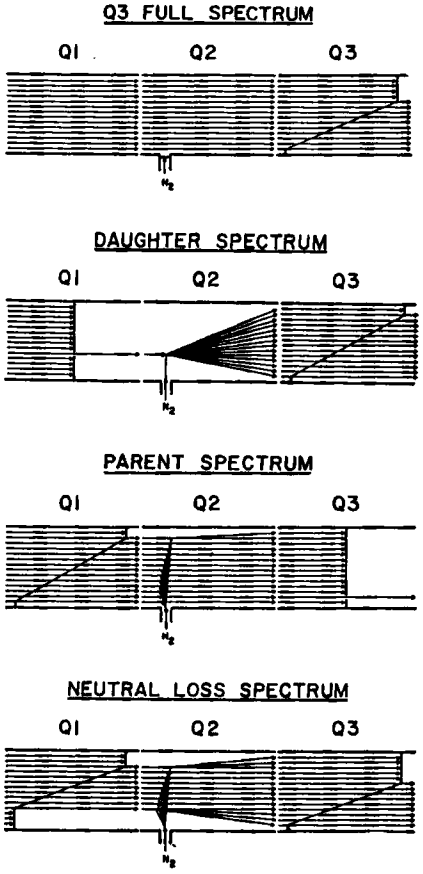

Figure 2. Operational modes of a tandem quadrupole mass spectrometer.

A **normal mass spectrum** can be obtained from either the first or second mass analyzer. A normal mass spectrum is typically obtained for pure compounds, usually to help select ions for a CAD experiment. A **daughter spectrum** is obtained when the first mass analyzer (Q1) selects ions of a particular mass (parent ion) for fragmentation by CAD in the collision quadrupole (Q2), and the second mass analyzer (Q3) scans the resulting fragments (the daughter ions). The resulting daughter spectrum will contain the fragment ions characteristic of the selected parent ion. The procedure is analogous to obtaining a normal mass spectrum of a component separated on-line by GC with MS, and can be used for identification of an analyte by mass spectral interpretation or by matching the daughter spectrum to that of an authentic sample. A **parent spectrum** is obtained when Q1 is scanned over a selected mass range to sequentially pass all ions produced in the source. These ions are then fragmented in Q2, and Q3 is set to pass a particular fragment ion mass. This results in a spectrum of parent ions which fragment to yield the selected daughter ion. This mode can be used to screen for a class of compounds that fragment to yield a common substructure. A **neutral loss spectrum** is obtained when both mass analyzers are scanned simultaneously with a particular mass offset between them. In this mode, ions of each mass in the selected mass range are passed sequentially by Q1 and allowed to fragment in Q2 as in a parent experiment. However, Q3 is scanned at the same rate as Q1 but with a selected mass difference which corresponds to the mass of the neutral fragment lost during CAD. Thus, a neutral loss experiment yields a spectrum of all parent ions which lose a selected neutral fragment. This mode is useful in screening for a class of compounds characterized by a common fragmentation pathway.

PREMISE OF THE MS/MS METHOD

It is useful to consider the structures of drug metabolites, their relationship to the original parent drug structure, and the functions of the neutral loss and parent scan modes of a tandem mass spectrometer. Since drug metabolites usually retain a portion of the parent drug molecule, they would be expected to lose some of the same neutral fragments and/or form some of the same daughter ions. If this does occur, both the neutral loss and parent modes of the MS/MS instrument can be effectively used to selectively screen a physiological sample for those species which contain the characteristic functional groups or substructures of the pure drug, without prior separation of the components in the mixture. The ions contained in the neutral loss and parent spectra would correspond to possible metabolites, and their daughter spectra could be obtained. With knowledge of both the fundamentals of drug metabolism and mass spectral interpretation, metabolite structures can thus be rapidly identified.

Summary of the MS/MS Procedure - The MS/MS procedure for metabolite identification can be summarized as follows:[5,6,16]

(1) A **normal chemical ionization (CI) mass spectrum** of the **pure drug** is obtained.
(2) **Daughter spectra** of the **pure drug** are obtained, selecting the protonated molecule, adduct ions, and abundant fragment ions for fragmentation, and noting the major neutral losses and abundant daughter ions.
(3) **Neutral loss spectra** of the **physiological sample** are obtained, selecting the major neutral losses of the pure drug observed in step 2. The ions contained in these spectra represent possible metabolites.

(4) **Parent spectra** of the **physiological sample** are obtained, selecting the abundant daughter ions observed in step 2. The ions contained in these spectra also represent possible metabolites.

(5) **Daughter spectra** of all the ions (possible metabolites) observed in steps 3 and 4 on the **physiological sample** are obtained. Metabolite structures can then be identified by comparison with daughter spectra of pure reference standards. Direct spectral interpretation without the use of reference standards can be used to postulate possible metabolite structures.

(6) Steps 3 and 4 are repeated on the physiological sample, selecting any new neutral losses and daughter ions observed from the daughter spectra of metabolites identified in step 5. This may permit the identification of other metabolites that have substructures similar to those of the previously identified metabolites, but not necessarily in the parent drug.

The numbered steps of the procedure listed above will be referred to later in this review.

METABOLITE IDENTIFICATION BY MS/MS

The MS/MS method has been successfully used for the systematic identification of known metabolite structures as well as new metabolite structures.[5,6,17,18] With this MS/MS approach, a wide variety of common classes of phase I metabolites (from oxidation, dealkylation, and hydrolysis of the parent drug) and phase II metabolites (from conjugation with glucuronic acid, sulfate, and amino acids) have been identified in crude serum and urine sample extracts. The results of these studies are reviewed below.

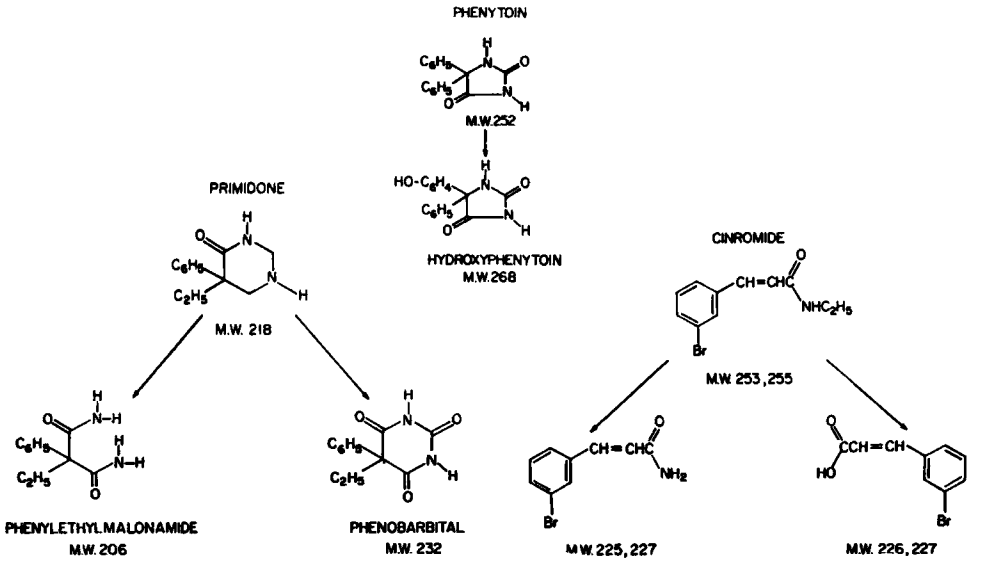

Figure 3. Major metabolites of the antiepileptic drugs primidone, cinromide, and phenytoin identified in urine and plasma by MS/MS.

Identification of Known Metabolite Structures - The validity and utility of MS/MS for the rapid identification of drug metabolites was first demonstrated by Perchalski et al.[5] With this MS/MS method, major metabolites of the antiepileptic drugs primidone, cinromide, and phenytoin were identified in urine and plasma (Figure 3). Major metabolites of carbamazepine have also been identified with this method.[6] By application of a knowledge of typical metabolic pathways, the major metabolites of these drugs were identified in only a few hours, with minimal sample preparation. Sample preparation for plasma samples involved the mild deproteinization followed by extraction, and for urine samples, hydrolysis and extraction to yield free, volatile species in a volatile solvent. Sample extracts were deposited in probe vials in 2 µl aliquots, evaporated, and introduced into the instrument on a direct insertion probe, where they were heated ballistically from 25 to 350°C in 2 minutes. With this procedure, metabolites have been identified at levels as low as 0.4 ng/µL in urine.[6,17]

Identification of New Metabolite Structures - The utility of this method for the identification of metabolites of a new antiepileptic drug, zonisamide, was recently reported.[16,17] In these studies, metabolite structures were deduced directly from the daughter spectra of potential metabolite molecular ions and fragments from an enzyme-hydrolyzed urine extract. No reference standards were used. The steps of the MS/MS method as they were applied for the identification of new metabolites of zonisamide are described below.

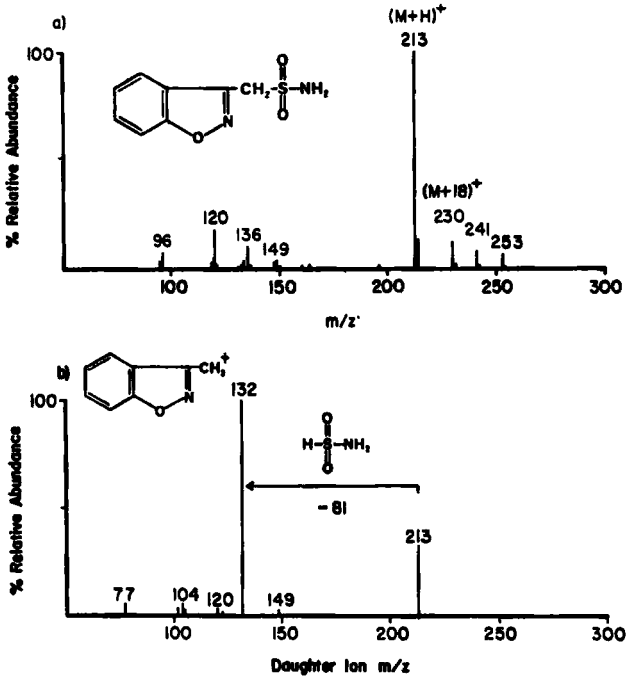

Figure 4. Normal and daughter spectra of pure zonisamide. (a) Normal positive CI mass spectrum. (b) Positive CI-CAD daughter mass spectrum of the protonated molecule, $(M+H)^+$.[16]

Figure 4a shows the spectrum resulting from step 1 of this method, a methane positive CI mass spectrum of pure zonisamide. The protonated molecular ion $[(M+H)^+]$ at m/z 213, methane adduct ions $[(M+C_2H_5)^+$ and

$(M+C_3H_5)^+$] at m/z 241 and 253, and fragment ions at m/z 96, 120 and 136 are present in the CI mass spectrum. For clarity, the daughter experiment (step 2) of only the protonated molecular ion of zonisamide (213^+) is presented. This spectrum (Figure 4b) indicates a major neutral loss of 81 mass units (u) to form an abundant daughter ion at m/z 132. The neutral loss spectrum (mass offset of 81 u) of the urine extract (step 3) is shown in Figure 5a. The spectrum contains abundant ions at m/z 229, 259, and 299, as well as 213 [$(M+H)^+$ of zonisamide]. The positive ion parent spectrum of m/z 132 of the urine extract (step 4) yielded, in addition to the ions characteristic of the original drug [the $(M+H)^+$ and $(M+C_2H_5)^+$ at m/z 213 and 241], ions at m/z 215, 229, and 255 (Figure 5b). Each of the abundant ions in these two spectra represent potential metabolites and were then chosen for the daughter ion experiment.

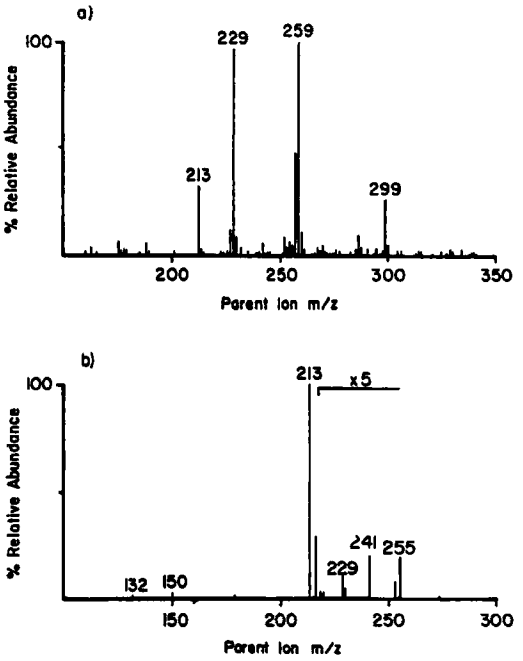

Figure 5. Neutral loss and parent spectra of enzyme-hydrolyzed urine extract. (a) Neutral loss (of 81) spectrum (b) Parent spectrum of m/z 132.[16]

Four possible metabolites of zonisamide identified in urine with this method are shown in Figure 6, along with the major CAD fragmentations observed in their daughter spectra. The metabolites A and B were identified by use of the neutral loss scan-daughter scan sequence, steps 3 and 5. This was possible because the metabolites retained the intact, unmetabolized sulfonamide portion of the parent drug, which was easily lost under CAD conditions (neutral loss of 81 u). Thus, metabolism occurred on the ring portion of the drug. The metabolites C and D were identified by the parent scan-daughter scan sequence, steps 4 and 5. These metabolites were the result of metabolism occuring on the sulfonamide portion of the drug, and thus, retained the ring substructure of the parent durg. Under CAD conditions, metabolites C and D formed an abundant daughter ion at m/z 132, corresponding to the ring substructure of the parent drug. No metabolite structures were assigned to ions at m/z 215 and 299 because no plausible metabolite structures could be deduced from their daughter spectra.

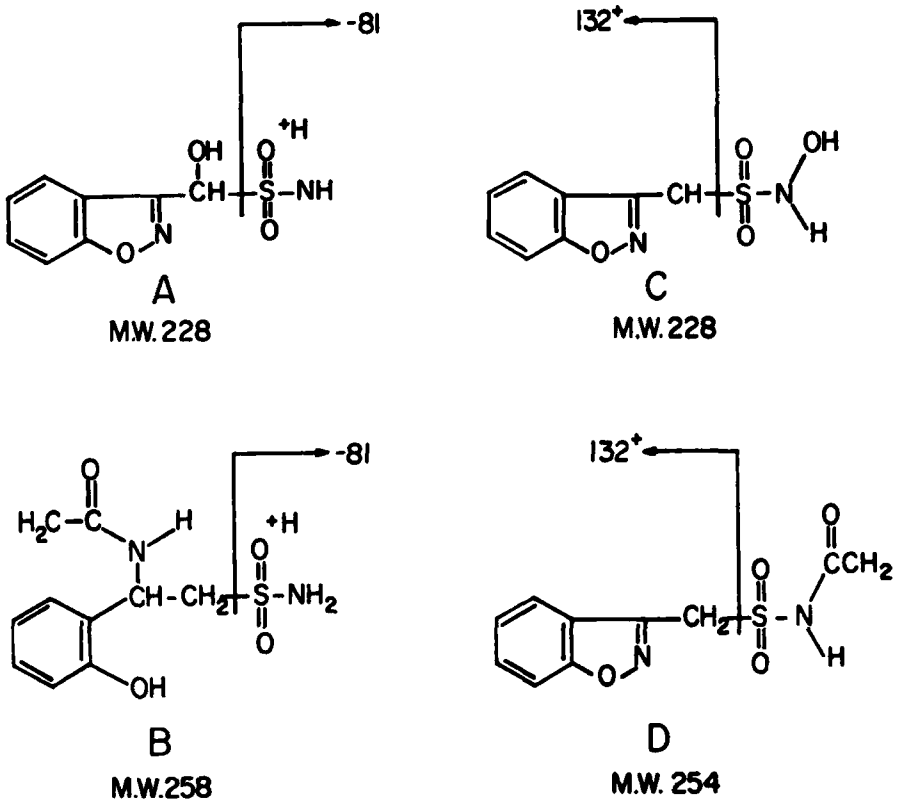

Figure 6. Metabolites of zonisamide identified by MS/MS and their characteristic fragmentations observed in the daughter spectra of their $(M+H)^+$ ions.[16]

Identifcation of Conjugated Drug Metabolites - The use of MS/MS for the identification of conjugated (phase II) metabolites was recently presented by Straub et al.[18] In their studies, conjugated metabolites such as the glucuronide of acetaminophen, the 4-sulfate ester of acetaminophen, and the 3-sulfate ester of dehydroisoandrosterone were identified in crude physiological samples by fast atom bombardment (FAB) MS/MS. The fragmentation behavior of a number of glucuronide, sulfate and glutathione (GSH) conjugates were investigated. O-glucuronides were found to undergo a neutral loss of 176 u, corresponding to the loss of glucuronic acid. This neutral loss was observed in both the positive and negative ion mode. Aryl sulfate esters are detected by a neutral loss of 80 u (SO_3) in both positive and negative ion modes. Alicyclic sulfate esters are best detected by the parent scan of m/z 97 (HSO_4^-) in the negative ion mode. Aryl-GSH conjugates are detected by the neutral loss of 275 u which corresponds to the mass of the peptide moiety, with cleavage of the S-peptide bond.

CONCLUSIONS AND PROSPECTS

The MS/MS technique offers a general, effective, and systematic method for the rapid identification of drug metabolites. By direct spectral interpretation of the step 5 daughter spectra, possible metabolite structures can be postulated without the use of pure reference standards.

The use of this MS/MS method should make drug metabolite identification studies more rapid, convenient, and economical, as well as open up new areas of application (e.g. pharmacokinetic studies, and rapid screening of individual patients for metabolic abnormalities that affect drug therapy). The primary use of this MS/MS method is to provide a rapid survey of the major metabolites contained in a physiological sample. It is unlikely that this method would provide a complete description of all the metabolites present in the sample. The rapid results of the MS/MS method may also be used to supplement classical metabolite identification techniques by indicating the best position for isotope incorporation into the drug for radiotracer experiments. The use of chromatography in combination with the MS/MS technique (GC/MS/MS and LC/MS/MS) for particularly difficult problems has significant potential. Investigation of techniques such as desorption chemical ionization[19], field desorption[20], laser desorption[21,22], secondary ion mass spectrometry[23], fast atom bombardment[18,24], and thermospray ionization[25-27] will almost certainly enhance the efficiency of this MS/MS technique for detection and identification of both phase I and phase II metabolites.

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Section VII. Worldwide Market Introductions

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Chapter 31. To Market, To Market - 1985

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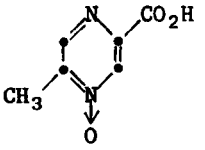
The new chemical entities (NCEs) for human therapeutic use introduced into the world marketplace for the first time in 1985 were about equal in number to those introduced in 1984.[1] Although not included in this chapter, a small, but increasing number of biologicals (interferons, hormones, etc.), derived through recombinant DNA research, are being launched. The United States topped the list of NCE originators in 1985, followed by Japan. West Germany had the most first time NCE introductions, followed closely by Japan and Italy.

A record number of NCEs were approved and marketed in the United States during 1985.[2] None of these, however, was a first time introduction. From the following compilation of 1985 first time worldwide NCE introductions, imipenem/cilastatin, levobunolol hydrochloride, quazepam and sulconazole nitrate had, by the year's end, been either marketed or approved for marketing in the US.

Acipimox (hypolipidemic)[3,4]

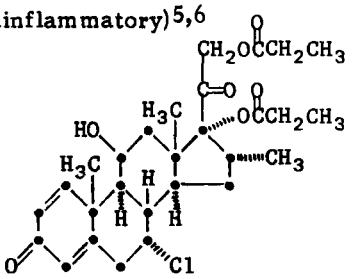
Country of Origin: **Italy**
Originator: **Erbamont**
First Introduction: **Italy**
Introduced by: **Erbamont**
Trade Name: **OLBETAM**



Acipimox is a hypolipidemic agent related to nicotinic acid with pronounced, long-lasting antilipolytic activity. In addition to its lipid lowering activity, it produces a beneficial elevation of the anti-atherogenic high density lipoprotein subfraction, HDL_2.

Alclometasone Dipropionate (topical antiinflammatory)[5,6]

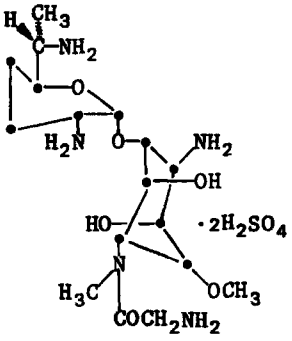
Country of Origin: **USA**
Originator: **Schering**
First Introduction: **Chile; W. Germany**
Introduced by: **Schering**
Trade Name: **VADERM**



Alclometasone dipropionate is a potent topical steroid useful in the treatment of atopic dermatitis and psoriasis.

Astromicin Sulfate (antibiotic)[7,8]

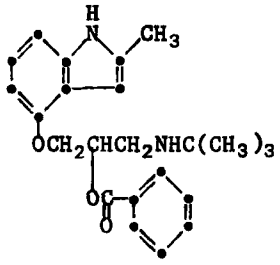
Country of Origin: **Japan**
Originator: **Kyowa Hakko**
First Introduction: **Japan**
Introduced by: **Kyowa Hakko**
Trade Name: **FORTIMICIN**



Astromicin sulfate is a pseudodisaccharide aminoglycoside antibiotic with a spectrum comparable to amikacin. It is active against many gentamicin and amikacin resistant bacteria expressing aminoglycoside-modifying enzymes, with the exception of aminoglycoside 3-acetyltransferase.

Bopindolol (antihypertensive)[9,10]

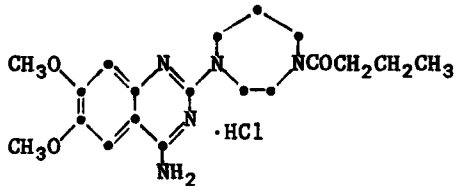
Country of Origin: **Switzerland**
Originator: **Sandoz**
First Introduction: **Switzerland**
Introduced by: **Sandoz**
Trade Name: **SANDONORM**



Bopindolol is a potent, long-acting, non-selective β-adrenergic blocker related to pindolol and useful in the treatment of hypertension. Unlike many other agents of this type, it appears to have negligible effects on serum lipoproteins.

Bunazosin Hydrochloride (antihypertensive)[11,12]

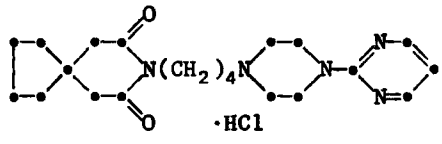
Country of Origin: **Japan**
Originator: **Eisai**
First Introduction: **Japan**
Introduced by: **Eisai**
Trade Name: **DETANTOL**



Bunazosin hydrochloride is a selective α_1-adrenergic antagonist related to prazosin and useful in the treatment of hypertension.

Buspirone Hydrochloride (anxiolytic)[13,14]

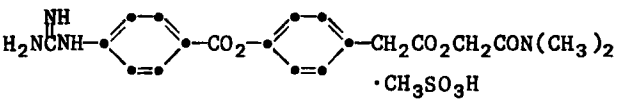
Country of Origin: **USA**
Originator: **Mead Johnson**
First Introduction: **W. Germany**
Introduced by: **Bristol**
Trade Name: **BESPAR**



Buspirone hydrochloride is an anxiolytic agent indicated for the management of anxiety disorders with or without accompanying depression. In contrast to the benzodiazepines, buspirone does not interact with alcohol, and lacks sedative, anticonvulsant, and muscle relaxant effects, and abuse potential.

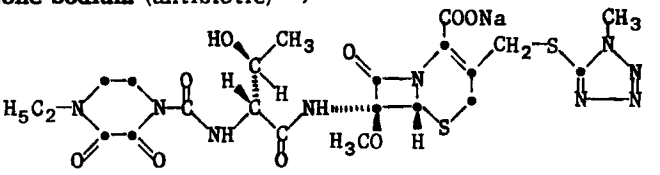
Camostat Mesylate (protease inhibitor)[15,16]

Country of Origin: **Japan**
Originator: **Ono**
First Introduction: **Japan**
Introduced by: **Ono**
Trade Name: **FOIPAN**



Camostat mesylate is a broad spectrum protease inhibitor useful in the treatment of chronic pancreatitis.

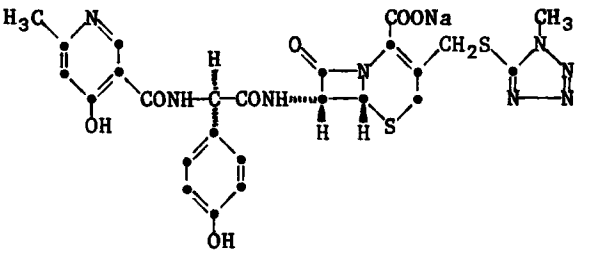
Cefbuperazone Sodium (antibiotic)[17,18]



Country of Origin: **Japan**
First Introduction: **Japan**
Trade Name: **TOMIPORAN; KEIPERAZON**
Originator: **Toyama**
Introduced by: **Toyama; Kaken**

Cefbuperazone sodium is a β-lactamase resistant, third generation cephalosporin with a serum half-life of about 1.5 hours.

Cefpiramide Sodium (antibiotic)[19,20]

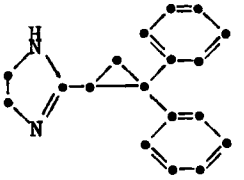


Country of Origin: **Japan**
First Introduction: **Japan**
Trade Name: **SEPATREN; SUNCEFAL**
Originator: **Sumitomo**
Introduced by: **Sumitomo, Upjohn; Yamanouchi, Banyu**

Cefpiramide sodium is a third generation cephalosporin with exceptionally good in vitro antipseudomonal activity. Its relatively long serum half-life (~4.5 hours) allows once to twice-daily dosing.

Cibenzoline (antiarrhythmic)[21,22]

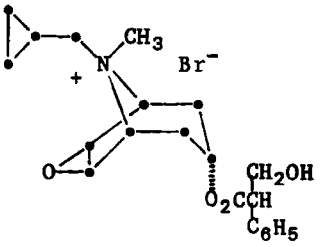
Country of Origin: **France**
Originator: **Hexachemie**
First Introduction: **France**
Introduced by: **UPSA**
Trade Name: **CIPRALAN**



Cibenzoline is a structurally novel, class I (quinidine-like) antiarrhythmic agent. It has additional properties of class III and class IV compounds, delaying repolarization and antagonizing the positive inotropic effects of calcium.

Cimetropium Bromide (antispasmodic)[23]

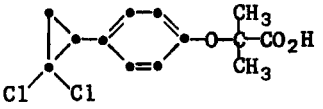
Country of Origin: **Italy**
Originator: **deAngelini**
First Introduction: **Italy**
Introduced by: **deAngelini**
Trade Name: **ALGINOR**



Cimetropium bromide is an analog of the anticholinergics methscopolamine, ipratropium and oxitropium bromides.[24] It is reported to be useful in the treatment of increased tone and motility of the gastrointestinal, biliary and urogenital tracts.

Ciprofibrate (hypolipidemic)[25,26]

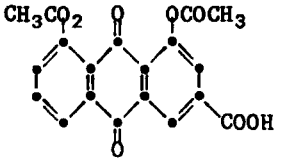
Country of Origin: **USA**
Originator: **Sterling-Winthrop**
First Introduction: **France**
Introduced by: **Sterling-Winthrop**
Trade Name: **LIPANOR**



Ciprofibrate is a potent, long-acting hypolipidemic agent related to clofibrate, bezafibrate and fenofibrate. It is effective in types IIa, IIb, III and IV hyperlipoproteinemias, and produces a beneficial elevation of the anti-atherogenic HDL.

Diacerein (antirheumatic)[27-29]

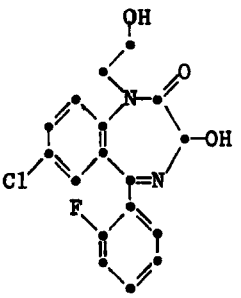
Country of Origin: **Italy**
Originator: **Proter**
First Introduction: **Italy**
Introduced by: **Proter**
Trade Name: **ARTRODAR**



Diacerein is a disease-modifying antirheumatic drug (DMARD), reported to be useful in the treatment of various inflammatory conditions, including osteoarthritis. It forms water soluble chelates with calcium and copper, acting ultimately to reduce the activity of lysosomal enzymes and decrease the formation of rheumatoid factor.

Doxefazepam (hypnotic)[30,31]

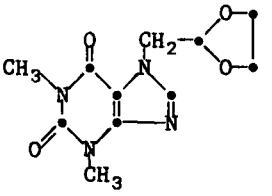
Country of Origin: **Italy**
Originator: **Schiapparelli**
First Introduction: **Italy**
Introduced by: **Schiapparelli**
Trade Name: **DOXANS**



Doxefazepam is a benzodiazepine hypnotic somewhat more potent than flurazepam.

Doxofylline (bronchodilator)[32,33]

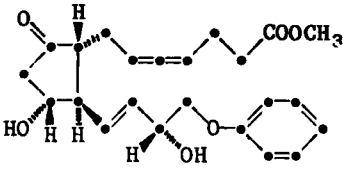
Country of Origin: **Italy**
Originator: **ABC S.p.A.**
First Introduction: **Italy**
Introduced by: **ABC S.p.A.**
Trade Name: **ANSIMAR**



Doxofylline, a xanthine bronchodilator related to theophylline, is useful in the treatment of asthma and similar bronchospastic disorders. It has minimal activity on intestinal smooth muscle and the cardiovascular system, and is reportedly devoid of the behavioral effects of theophylline.

Enprostil (antiulcer)[34,35]

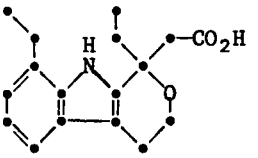
Country of Origin: **USA**
Originator: **Syntex**
First Introduction: **Mexico**
Introduced by: **Syntex**
Trade Name: **GARDRIN**



Enprostil is one of three, orally-active prostaglandins (see misoprostol and rosaprostol) launched this year for the treatment of gastric and duodenal ulcers. An allenic PGE_2 derivative, enprostil is both antisecretory (acid, gastrin) and cytoprotective.

Etodolac (antiinflammatory)[36-38]

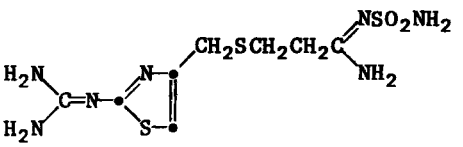
Country of Origin: **USA**
Originator: **Ayerst**
First Introduction: **Colombia; United Kingdom**
Introduced by: **Ayerst**
Trade Name: **LODINE**



Etodolac (etodolic acid) is a non-steroidal antiinflammatory/analgesic agent useful in the treatment of various inflammatory conditions, including rheumatoid and osteoarthritis.

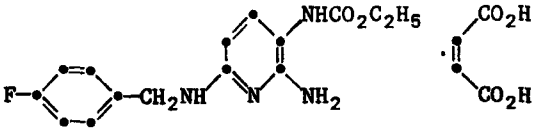
Famotidine (antiulcer)[39,40]

Country of Origin: **Japan**
Originator: **Yamanouchi**
First Introduction: **Japan**
Introduced by: **Yamanouchi**
Trade Name: **GASTER**



Famotidine is a histamine H_2-antagonist more potent than cimetidine and ranitidine. Administered once or twice daily, it is useful in the treatment of gastric, duodenal and anastomotic ulcers, upper gastrointestinal tract hemorrhage, reflux esophagitis and Zollinger-Ellison syndrome. Like ranitidine, it is lacking in antiandrogenic effects.

Flupirtine Maleate (analgesic)[41,42]

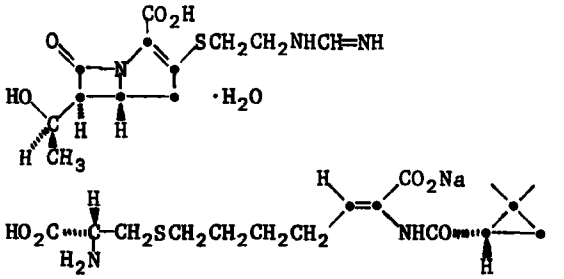


Country of Origin: **W. Germany** Originator: **Chemiewerk Homburg (Degussa)**
First Introduction: **W. Germany** Introduced by: **Chemiewerk Homburg (Degussa)**
Trade Name: **KATADOLAN**

Flupirtine maleate is a centrally-acting, non-addicting analgesic agent somewhat more potent than aspirin, paracetamol and pentazocine. It is reported to be useful in the treatment of postoperative and dental pain.

Imipenem/Cilastatin (antibiotic)[43,44]

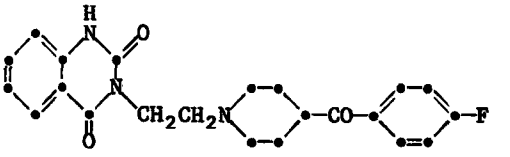
Country of Origin: **USA**
Originator: **Merck**
First Introduction: **W. Germany**
Introduced by: **Merck**
Trade Name: **ZIENAM**



Imipenem is a chemically stable thienamycin derivative with an antibacterial activity that is broader in spectrum and of greater potency than most of the third generation cephalosporins. Combination with cilastatin, an inhibitor of renal brush-border dehydropeptidase-I, increases both urinary and plasma levels of imipenem.

Ketanserin (antihypertensive)[45,46]

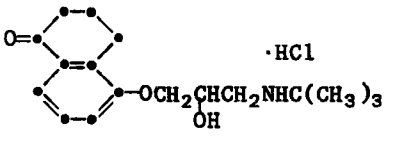
Country of Origin: **Belgium**
Originator: **Janssen**
First Introduction: **Argentina**
Introduced by: **Janssen**
Trade Name: **SEREFREX**



Ketanserin is an antihypertensive agent with antagonist properties at both the serotonin-S_2 and α_1-adrenergic receptors. Its mechanism of action, while still controversial, may involve interactions at both of these loci.

Levobunolol Hydrochloride (antiglaucoma)[47,48]

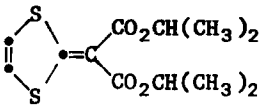
Country of Origin: **USA**
Originator: **Warner Lambert**
First Introduction: **W. Germany**
Introduced by: **Allergan**
Trade Name: **VISTAGEN LIQUIFILM**



Levobunolol hydrochloride is a non-selective β-adrenergic blocker useful in the treatment of glaucoma. Worldwide, it represents the sixth β-blocker to be launched for this indication.

Malotilate (hepatoprotective)[49,50]

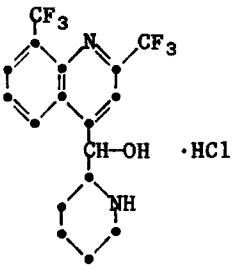
Country of Origin: **Japan**
Originator: **Nihon Nohyaku**
First Introduction: **Japan**
Introduced by: **Daiichi Seiyaku**
Trade Name: **KANTEC**



Malotilate is a hepatoprotective agent reported to be effective in the treatment of cirrhosis, chronic hepatitis and similar liver disorders. It appears to improve hepatic function by increasing blood and bile flow and improving protein synthesis.

Mefloquine Hydrochloride (antimalarial)[51]

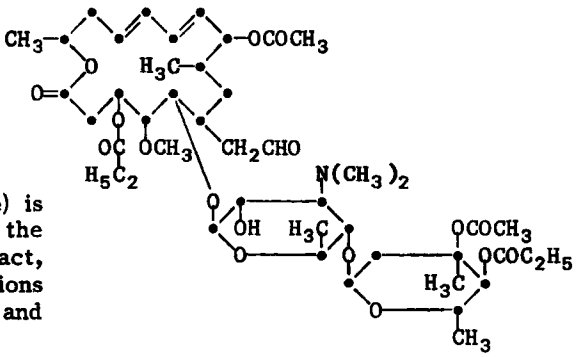
Country of Origin: **USA**
Originator: **Walter Reed**
First Introduction: **Thailand**
Introduced by: **Hoffmann-La Roche**
Trade Name: **FANSIMEF**



Mefloquine hydrochloride is an erythrocytic schizonticide useful, in combination with sulfadoxine and pyrimethamine, in the prophylaxis and treatment of malaria. Administered once weekly, the combination is effective against pathogens resistant to other agents.

Miokamycin (antibiotic)[52,53]

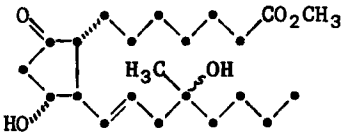
Country of Origin: **Japan**
Originator: **Meiji Seika**
First Introduction: **Japan**
Introduced by: **Meiji Seika**
Trade Name: **MIOCAMYCIN**



Miokamycin (midecamycin acetate) is a macrolide antibiotic useful in the treatment of respiratory tract, dermatological and surgical infections due to gram-positive bacteria and mycoplasmas.

Misoprostol (antiulcer)[35, 54-56]

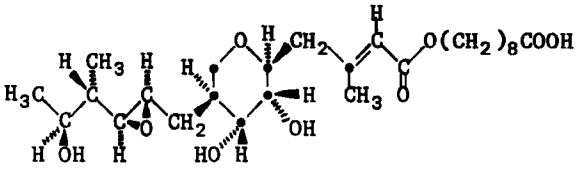
Country of Origin: **USA**
Originator: **Searle**
First Introduction: **Mexico**
Introduced by: **Searle**
Trade Name: **CYTOTEC**



(±16 R,S)

Misoprostol is an orally-active PGE_1 analog useful in the treatment of gastric and duodenal ulcers. As with other agents of this type, misoprostol may prove useful as a gastric cytoprotective when administered concurrently with irritating drugs such as nonsteroidal antiinflammatories.

Mupirocin (topical antibiotic)[57-59]

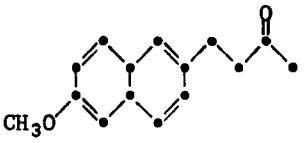


Country of Origin: **United Kingdom** Originator: **Beecham**
First Introduction: **United Kingdom** Introduced by: **Beecham**
Trade Name: **BACTROBAN**

Mupirocin (pseudomonic acid A) is an antibiotic produced by Pseudomonas fluorescens. It is useful in the treatment of dermal infections, especially those involving S. aureus and S. epidermidis.

Nabumetone (antiinflammatory)[60-63]

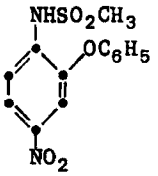
Country of Origin: **United Kingdom**
Originator: **Beecham**
First Introduction: **Ireland**
Introduced by: **Beecham**
Trade Name: **RELIFEX**



Nabumetone is a non-acidic, nonsteroidal antiinflammatory agent formally related to naproxen. Its main circulating metabolite is 6-methoxy-2-naphthylacetic acid (α-nornaproxen). Administered once daily ($T_{1/2} \approx 30$ hrs), nabumetone is reported to be effective in the treatment of rheumatoid and osteoarthritis.

Nimesulide (antiinflammatory)[64-67]

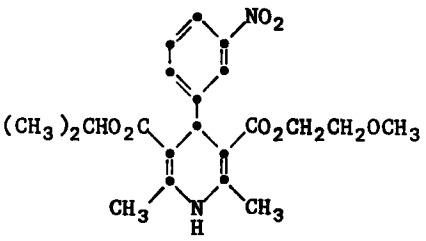
Country of Origin: **USA**
Originator: **Riker**
First Introduction: **Portugal**
Introduced by: **Labs Biofarma**
Trade Name: **MESULIDE**



Nimesulide is a nonsteroidal antiinflammatory/analgesic agent useful in the treatment of rheumatoid arthritis, as well as acute inflammation such as that induced by periodontal surgery or urinary tract infections.

Nimodipine (cerebral vasodilator)[68,69]

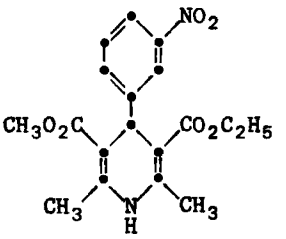
Country of Origin: **W. Germany**
Originator: **Bayer**
First Introduction: **W. Germany**
Introduced by: **Bayer**
Trade Name: **NIMOTOP**



Nimodipine is a cerebral vasodilating calcium antagonist related to nifedipine. It is indicated for the prophylaxis and treatment of neurological deficits due to cerebral vasospasm after subarachnoid hemorrhage.

Nitrendipine (antihypertensive)[70,71]

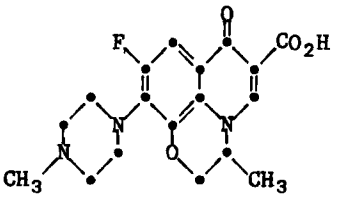
Country of Origin: **W. Germany**
Originator: **Bayer**
First Introduction: **W. Germany**
Introduced by: **Bayer**
Trade Name: **BAYOTENSIN**



Nitrendipine is a vasodilating, dihydropyridine calcium antagonist. Its prolonged therapeutic effect in comparison to nifedipine makes it useful in the once to twice daily treatment of hypertension.

Ofloxacin (antibacterial)[72,73]

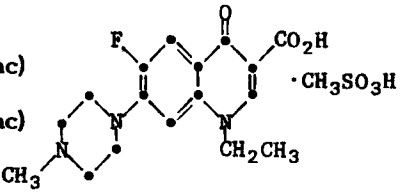
Country of Origin: **Japan**
Originator: **Daiichi Seiyaku**
First Introduction: **W. Germany**
Introduced by: **Hoechst AG**
Trade Name: **TARIVID**



Ofloxacin is an antibacterial agent with increased potency in comparison to the prototype third generation quinolone, norfloxacin.[24] It has a broad spectrum of activity against gram-positive and gram-negative bacteria and is useful in the treatment of kidney, genitourinary, and upper respiratory tract infections.

Pefloxacin Mesylate (antibacterial)[74,75]

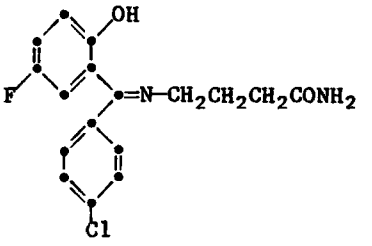
Country of Origin: **France**
Originator: **Roger Bellon (Rhone-Poulenc)**
First Introduction: **France**
Introduced by: **Roger Bellon (Rhone-Poulenc)**
Trade Name: **PEFLACINE**



Pefloxacin mesylate is a broad-spectrum, third generation quinolone antibacterial agent useful in the treatment of infections due to a variety of organisms, including methicillin-resistant S. aureus. A major metabolite of pefloxacin is norfloxacin.[24]

Progabide (anticonvulsant)[76-78]

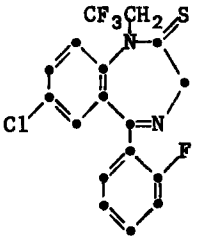
Country of Origin: **France**
Originator: **Synthelabo**
First Introduction: **France**
Introduced by: **Synthelabo**
Trade Name: **GABRENE**



Progabide is a broad-spectrum anticonvulsant agent indicated for the treatment of a wide variety of seizure disorders. Synthesized as a GABA-prodrug, anticonvulsant activity appears due to the parent drug and its acid metabolite, as well as to GABAmide and GABA liberated therefrom.

Quazepam (hypnotic)[79-80]

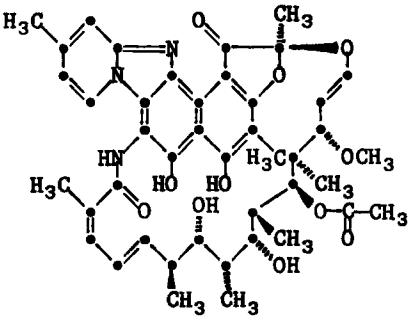
Country of Origin: **USA**
Originator: **Schering**
First Introduction: **Netherlands**
Introduced by: **Schering**
Trade Name: **SELEPAM**



Quazepam is a rapidly acting, short term hypnotic reported to exhibit a low incidence of side effects.

Rifaximin (antibiotic)[81,82]

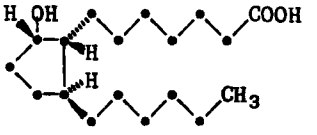
Country of Origin: **Italy**
Originator: **Alfa**
First Introduction: **Italy**
Introduced by: **Schiapparelli**
Trade Name: **RIFACOL**



Rifaximin is a non-absorbable rifamycin derivative useful in the treatment of intestinal diarrhea. It displays good activity against a wide spectrum of bacteria, including Salmonella spp., S. aureus, and E. coli.

Rosaprostol (antiulcer)[83,84]

Country of Origin: **Italy**
Originator: **Instituto Biochemico Italiano**
First Introduction: **Italy**
Introduced by: **Instituto Biochemico Italiano**
Trade Name: **ROSAL**

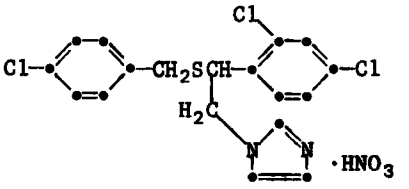


(±5 R,S)

Rosaprostol is an antisecretory and cytoprotective prostaglandin analog useful in the treatment of gastric and duodenal ulcers. Considerably less potent than enprostil or misoprostol, it is reported to lack the typical gastrointestinal and cardiovascular side effects of PGE derivatives.

Sulconazole Nitrate (topical antifungal)[85-87]

Country of Origin: **USA**
Originator: **Syntex**
First Introduction: **United Kingdom**
Introduced by: **ICI**
Trade Name: **EXELDERM**



Sulconazole nitrate is an imidazole antifungal agent closely related to econazole. It is effective in the topical treatment of a broad spectrum of superficial fungal infections.

Thymopentin (immunomodulator)[88-90]

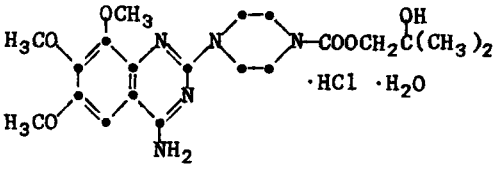
Country of Origin: **Belgium**
Originator: **Janssen**
First Introduction: **Italy**
Introduced by: **Italfarmaco; Cilag**
Trade Name: **SINTOMODULINA; TIMUNOX**

Arg-Lys-Asp-Val-Tyr

Thymopentin is a synthetic, immunomodulating pentapeptide, representing the active fragment of the thymus hormone, thymopoietin. Thymopentin induces phenotypic differentiation and maturation of T-cells. It appears of value in the treatment of primary immune deficiency, rheumatoid arthritis, atopic dermatitis, sarcoidosis and a variety of other diseases with manifestations of disturbed immune balance.

Trimazosin Hydrochloride (antihypertensive)[91,92]

Country of Origin: **USA**
Originator: **Pfizer**
First Introduction: **W. Germany**
Introduced by: **Pfizer**
Trade Name: **SUPRES**



Trimazosin hydrochloride is a selective α_1-adrenergic antagonist related to prazosin, bunazosin, and terazosin.[1] It is reported to be longer acting than prazosin, without first dose hypotensive effects.

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